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Isolation of Mycobacterium Tuberculosis from Clinical Materials by Pepsin Digestion and Interface Concentration with Pentane

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ISOLATION OF MYCOBACTERIUM TUBERCULOSIS FROM CLINICAL MATERIALS BY PEPSIN DIGESTION AND INTERFACE CONCENTRATION WITH PENTANE

by

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LIFE

Sister M. Imogene Palen was born in Modoc, Kansas on April 25, 1914. She completed Girls Catholic High School in Hays, Kansas, in 1931. Sister was professed as a Sister of Saint Agnes in Fond du Lac, Wisconsin in 1935. She taught in parochial schools of Milwaukee and attended Marquette University where she received the Bachelor of Science degree in 1941. Sister M. Imogene taught mathematics and science at St. Mary Springs Academy until 1946. At this time she studied bacteriology at Marquette University Medical School and a year later undertook duties in this field at St. Agnes Hospital and Marian College, Fond du Lac.

Sister M. Imogene entered the Department of Microbiology at Loyola University Graduate School in September, 1953, and continued there without interim until the present date.

She is co-author of : Variations and Spontaneous Mutations in the Genus Listeria in Respect to Flagellation and Motility. Leifson, E. and Palen, M.I., 1955, J. Bacteriol. 70: 233-240.

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Chapter I

INTRODUCTION

Three general methods are used, singly or in combination for the diagnosis of tuberculosis in human beings. Direct smear and acid fast staining is the quickest and simplest of these but by itself the method is not very reliable. Guinea pig inoculation has the disadvantages of being expensive, having a long diagnostic time, and not highly reliable. It seems generally agreed that artificial culture methods are best, but still far from perfect. In this dissertation is presented a method of isolation of tubercle bacilli from human sputum which is simple to perform and appears to be superior to any other method in use at present.

The number of tubercle bacilli in the sputum of an active case of tuberculosis may be very small and impossible to find unless the sputum is highly concentrated. The first step is to destroy the mucus. Various acids and alkalies are commonly employed for this purpose, but a considerable proportion of the tubercle bacilli are usually killed in the process. Enzymes such as pepsin and trypsin have been used to digest the mucus and are less lethal for

the tubercle bacilli than the chemicals mentioned. For several reasons explained later, pepsin slightly acidified with citric acid seems to be the enzyme system of choice. Having eliminated the mucus, the next problem is the concentration of the bacilli. This may be accomplished in several ways such as centrifugation, and layering. The last is a method of concentration in a layer at an interface of water and a hydrocarbon. The most efficient of these appears to be interface concentration by the use of pentane. The third problem is the extraneous bacteria in the sputum. Where acids or alkalies are used to destroy the mucus most of these bacteria are also killed. This is not the case where enzymes are used. The pentane, however, does not concentrate the extraneous bacteria to the same extent as the tubercle bacilli and, in fact, is quite lethal for them.

STATEMENT OF PROBLEM

To develop a method for the isolation of tubercle bacilli from human sputum which is more efficient or productive than the methods in use at present.

To review past work along this line.

To present comparative data obtained with the new and old methods.

Chapter II

LITERATURE REVIEW

Our problem is threefold and the review is organized in this way. The processes are not always distinct, hence digestion and decontamination are frequently overlapping procedures.

Digesting Agents

Griffith (1914) used sodium hypochlorite which was called antiformin. To 1 part of sputum he added 1 part of 10% antiformin. After 20 minutes at room temperature he centrifuged, decanted, washed and recentrifuged the sediment. Later Griffith (1916) found the washing unnecessary.

Petroff (1915) introduced the use of sodium hydroxide. At this time he used 3% in a 1:1 ratio with sputum. Lurie (1923) used 4% NaOH which is still used extensively today.

Acid digestants were used by Lowenstein (1924). He preferred 3% HCl and 6% H_2SO_4. Corper (1927) and (1928) used 6% H_2SO_4 in 1:1 ratio with sputum for 30 minutes, although the stated that HCl and NaOH were satisfactory. Na_2CO_3 and NH_4OH were not satisfactory to Corper. In (1929) Corper found 6% oxalic acid more satisfactory than 6% H_2SO_4.

In (1946) Corper abandoned acid digestion in favor of 10% Na_3PO_4. A greater proportion of tubercle bacilli survived with the 10% Na_3PO_4 than with any agent he had used.

Saelhof (1924) recommended an alum hydroxide cream composed of 1% NH_4OH and 1% $AlNH_4(SO_4)_2$ to precipitate the tubercle bacilli in treated sputum. He washed the precipitate until the water showed no residue on evaporation. This method was also used for cerebrospinal fluid and pleural fluid.

Steenken (1940) described a precipitating protein prepared from Berkefeld filtered pleural fluid which was alcohol precipitated and alcohol washed. The protein was resuspended in 50% alcohol (50 mg dry wt/ml), and 1 ml of the solution added to 100 ml of urine precipitated the tubercle bacilli for further processing.

Sometimes decontamination seemed to be more of an objective than recovery of tubercle bacilli. Oliver and Rensser (1942) and Cameron and Castles (1945) used "chlorox" which is 15% NaOH and 20% NaOCl. It left no extraneous material, which they considered highly desirable, but they admitted it killed the tubercle bacilli.

Spendlove, Cummins, and Patnode (1949) made a study showing the relative toxicity of various digestants for tubercle bacilli. A 3 week culture of H37Rv was used in

dilutions to give 10^{-6} mg of tubercle bacilli in each tube of Lowenstein-Jensen (L-J) medium. The inocula were exposed to the digestants in a 1:1 ratio at 37 C. The culture tubes were inoculated from each digestion as soon as the digestant was added (control), after 40 minutes, 24 hours, 48 hours, 72 hours. The total number of colonies were counted after 5 weeks incubation.

	Colonies Counted					
	10% Na_3PO_4	4% NaOH	2.5% $(NH_4)_2CO_3$	5% H_2SO_4	50% Antiformin	3% HCl
Control	173	68	48	93	79	83
40 min.	96	60	0	27	0	0
24 hrs.	94	6	0	0	0	0
48 hrs.	91	5	0	0	0	0
72 hrs.	71	0	0	0	0	0

Because of the possible protection that sputum would give to tubercle bacilli, the experiment was repeated with 10 positive sputa homogenized for 2 hours with a paint shaker. Inoculations were made after 20 and 40 minutes, 2 and 24 hour periods with 0.1 ml amounts of the digestions for each. A summary of their data on this experiment is presented.

	Total Colonies on 10 Tubes					
	4% NaOH	10% Na_3PO_4	5% Ac. Oxalic	5% H_2SO_4	2.5% $(NH_4)_2CO_3$	3% HCl
20 min.	*/	*/	/	*/	/	400
40 min.	*	*	*	*	*	140
2 hrs.	*	*	*	1000	1000	0
24 hrs.	1000	1000	1000	0	0	0

* Innumerable colonies of acid fast bacilli

/ Contamination

Smith,Nishikan, Golden, Hoyt, Guss, Kloetzel (1950) used detergents for disinfection in general and for isolation of tubercle bacilli. Armeen 14D (a detergent) was lethal to the organisms while 141 other detergents studied including nonionic, cationic, and anionic compounds were not lethal.

In Japan, Nosaya, and Soeda (1951), used two kinds of detergents, oxvan (dimethyl-lauryl-benzyl-oxyethyl-ammonium chloride) and laboran (cetyl-diethyl-methyl-ammonium methosulphate). They treated the sputum with each detergent in 0.5% solution for ½, 1, 2, and 24 hours, and obtained good growth on Petragnani and blood agar after incubation for 3 weeks with no apparent differences due to the time of exposure. Ogawa (1951) got better results with 0.5% laboran than with 6% H_2SO_4. Minoru (1953) homogenized sputum with a filtrate of Bacillus fulminous and 0.5% NaOH.

This was followed by treatment with 0.5% detergent (germinol), and the media inoculated directly.

Ajello (1951) found that most digestants were lethal to fungi. He used 4% NaOH for 10 minutes in a paint shaker, 4% NaOH without mechanical shaking, 5% H_2SO_4, and 10% Na_3PO_4 as digestants on pooled sputum seeded with Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Cryptococcus neoformans, Geotrichum candidum, Histoplasma capsulatum, and Nocardia asteroides. Exceptions to the lethal effects of the digestants on these fungi were few. N. asteroides was isolated from sputum in every test using Na_3PO_4 but it didnot survive the other digestants. C. albicans was recovered in every case with the H_2SO_4 procedure, but it did not survive the other digestants. C. neoformans alone survived digestion with NaOH.

Yegian and Budd (1952) showed that 50% of the tubercle bacilli were killed in the first 5 minutes by the 4% NaOH method. They suggested that the "negative" culture which is reported should be interpreted with an appreciation of the limitations of present laboratory methods for the culture of tubercle bacilli. They discussed the significance of this in regard to specimens with just a few tubercle bacilli.

Darzins (1952) described a shake-precipitation

method. He used a combination of 1% Na_3PO_4 and 1% NaOH as the digestion agent,and calcium phosphate to precipitate protein and carry the tubercle bacilli along with the precipitate. With his procedure he obtained 61.4% positive cultures in contrast to 48.5% positives with the 6% H_2SO_4 method.

Browning (1953) used a solution of Teepol, which he described as a detergent of sodium alkyl sulphates, to homogenize and decontaminate sputum.

Hirsch (1954) investigated zephiran as a decontaminant which is a cationic detergent, a mixture of high molecular, alkyldimethyl-benzyl-ammonium chlorides. He suggested its use as a bactericidal agent for sputum contaminants. In this laboratory we had already been working with zephiran and enzymes for sometime previous to Hirsch's publication.

Almost at the same time was anote by Saxhola (1954) Discussing the use of a pancreatin-desogen solution. The desogen, a quaternary ammonium compound, was described as methyl-phenyl-dodecyl-trimethyl-ammonium-methosulphate. In (1955) he published a complete report of 3,818 sputum specimens. He concluded that long periods of exposure were not deleterious to the tubercle bacilli.

Gray, Clarke, and Johnstone (1954) proposed the

use of black mice (strain C58) in combination with culture to be much more effective than the culture alone. In a later paper (1954 a) they claimed that in the usual digestion procedures 80 to 100% of the tubercle bacilli are killed. They considered treatment with 4% NaOH for 30 minutes or 10% Na_3PO_4 for 24 hours to be least lethal.

Pelzer, Chaves, and Widelock (1954) used 10% Na_3PO_4 as a transport digestant for sputa and gastric specimens as "treatment in transit" where the time of transportation was longer than 24 hours. Duplicate halves of the specimen were untreated until arrival in the laboratory when they were processed with 4% NaOH. They found the recovery of tubercle bacilli the same by both methods, but much less contamination with the use of 10% Na_3PO_4.

From the studies cited it is apparent that most procedures used today are toxic to tubercle bacilli. Bader (1954) compared treated and untreated specimens by means of guinea pig inoculation to demonstrate the toxicity of present day procedures.

At various times penicillin has been used as a decontaminating agent following a digestion procedure. Kirby (1947) believed that in the presence of penicillin tubercle bacilli undergo partial lysis. Fifty to 100

units of penicillin per ml in oleic acid albumin agar were not inhibitory to the tubercle bacilli, but the same amount of penicillin in broth media in the presence of "tween 80" was inhibitory to tubercle bacilli.

Abbott (1951) recommended the addition of 100-200 units of penicillin to homogenized sputum concentrates just before inoculation. He reported that the contamination was reduced 50%. He also found the rate of growth and the volume of growth of the tubercle bacilli diminished slightly following penicillin treatment. Abbott did not consider the final recovery "significantly affected."

Fallin, Patnode, and Hudgins (1952) found that 100 to 400 units of penicillin per ml of homogenized sputum significantly reduced the number of viable tubercle bacilli in the sputum concentrates in direct proportion to the amount of antibiotic used.

Frobisher and Sommermeyer (1953) considered that viscous sputum gives considerable protection to tubercle bacilli and other contaminants present. Ethyl alcohol and isopropyl alcohol (rubbing alcohol) in 70% concentrations were not found to be effective disinfectants for any of the organisms in sputum. They suggest that this is important when oral thermometers are disinfected.

Enzyme Digestion

Berg (1932) did a search of the literature and found pancreatic enzymes used as digestants as far back as 1903. He challenged the assumption that pancreatic enzymes were nonlethal to tubercle bacilli. From his studies he concluded that by weight ½ of the tubercle bacilli were autolyzed within 4 days in the presence of pancreatic enzymes.

McNamara (1935) used a final concentration of 0.3% pepsin and 0.6% HCl in sputum and incubated for 12 hours. He neutralized with N/1 NaOH and then centrifuged the digest for 1 hour.

Sullivan and Sears (1939) prepared a paste using 0.5 gm of papain with 50 ml of sputum and allowed it to digest for 10 to 15 minutes. They centrifuged the digest and treated the sediment further with 4% NaOH prior to cultivation.

Vogt, Zappasodi, and Long (1940) used 0.1% trypsin for digestion at 56 C for 2 hours. Haynes (1942) prepared a digestant of 0.5% trypsin and added 0.7% of N/1 NaOH which gave the digestant a pH of 8.5. Haynes filtered (Seitz) the reagent and used a 1:1 ratio with sputum. The mixture was incubated at 37 C for ½ hour,

centrifuged at 3000 r.p.m. for five minutes and the sediment used for acid fast stains. For guinea pig inoculation it was further treated with 5% oxalic acid.

Schwarting (1945) and (1948) showed that gastric juice was toxic for tubercle bacilli dependant upon the length of exposure and the temperature. She used artificial gastric solution and normal gastric juice with controlled inocula of tubercle bacilli. Refrigeration of pooled specimens did not prevent lethal effects on tubercle bacilli.

Kramer (1946) found that after 10 to 21 hours of exposure of tubercle bacilli to gastric juice the organisms were noninfective for guinea pigs nor could they be recovered on artificial media.

Sedallian and Carraz (1947) used papain. We repeated their experiment which called for incubation at 60 C and found the same liquefaction of sputum at that temperature with a water control.

The effect of gastric contents on tubercle bacilli is indicative of the deleterious effects of pepsin and HCl as a digesting agent. Vincent and Birge (1947) showed that tubercle bacilli survive in gastric contents about 5 days at neutral pH. At pH 6 the contaminants were held down yet growth was favorable for tubercle bacilli.

Vandiviere, Smith, and Sunkes (1952) blamed the

high acidity for the toxic effect of gastric juice on tubercle bacilli. They evaluated methods for collection. The best was to collect the gastric contents in 300 ml of sterile water and add two "pHydrion" (7.4) capsules immediately.

Reuss (1952) showed in his investigations that tubercle bacilli subjected to gastric juice from 1 to 7 days were still demonstrable by culture and animal inoculation.

Rice and Rowan (1953) developed a microculture technique for the isolation of tubercle bacilli. One volume of sputum was mixed with 2 volumes of 2.5% pancreatin and incubated at 37 C for ½ hour. The digested sputum was then streaked on a coverslip, dried, and further treated with 8% HCl for 6 minutes. The preparation was carefully rinsed in water and incubated in liquid media.

In chronic bronchitis May (1953) proved the need for homogenized sputum for a true representation of pathogens that may be in the specimen. He suggested Rawlins' (1953) method which used 1% pancreatin at pH 7.6 held at 37 C until liquefaction was complete. He found that this procedure was also useful for recovery of the common respiratory pathogens other than tubercle bacilli.

Ambert (1953) used 3 ml of an aqueous suspension of tubercle bacilli with 100 mg of pepsin (dry) and 2 drops of concentrated HCl to test the toxicity of a pepsin-HCl

digestion procedure for sputum. Those inocula which had been exposed more than 10 hours before culture were slower to produce colonies than the controls.

Hain (1954) suggested an alkaline buffer to neutralize the lethal effects of the enzyme activated by an acid milieu contained in gastric juice. He used 1% KH_2PO_4 and 2.5% Na_3PO_4 with 0.00128 % broxcresol purple indicator. He found 30 ml of the indicator-buffer was a sufficient amount to eliminate toxicity for the tubercle bacilli when transportation or storage of gastric specimens was necessary.

Saxholm (1954, 1955a, 1955b) used 1% pancreatin for digestion and a quaternary ammonium detergent for decontamination. The treatment showed little harmful effect on the tubercle bacilli in 3,818 specimens treated, but he refers to a "softening of media" with large inoculation of the digest. Our experience with the same difficulty caused us to turn to pepsin.

Concentration Methods

Recently membrane filters have given a lot of promise in those specimens that can be adapted to the technique. Trompke and Kroger (1952) found membrane filtration much more efficient than centrifugation for urine specimens. Morgante and Murray (1955) got more positives by membrane filtration of spinal fluid than by direct inoculation on Lowenstein-Jensen media.

The use of hydrocarbons as concentrating agents dates as far back as (1909) when Lange and Nitsche used alkali and ligroin. Ligroin is a petroleum distillate with specific gravity of 0.715. Bernhardt (1909) used antiformin and ligroin. Loeffler (1910) used antiformin and chloroform. Kinyoun (1915) modified Uhlenhuth's method by treating the specimen with sodium hypochlorite followed by concentration with ligroin.

Andrus (1924) used chloroform to layer the organisms at the bottom of the tube.

Felsen (1930) floated tubercle bacilli in the homogenate by the use of sodium chloride in 2% concentration. The mixture was allowed to stand in the refrigerator overnight, and the tubercle bacilli were skimmed from the surface.

Reed and Rice (1931) studied the behavior of acid fast and many other bacteria in oil and water mixtures.

All the bacteria remained in the aqueous phase except the acid fast organisms. They worked with many oils, hydrocarbons, and other compounds including carbon tetrachloride, ether, xylene, kerosene, benzene, petroleum, chloroform, carbon bisulphide, butyl and amyl alcohol.

Pottenger (1931) and (1939) used xylene, ligroin, chloroform, carbon disulfide, and gave convincing evidence of the efficiency of these agents to concentrate acid fast bacilli.

In a review of evaluation of methods up to 1951 Smith(1951) recalled his own findings of the efficiency of "hydrocarbon flotation". Slopek (1953) also found flotation to be an efficient method for isolation of tubercle bacilli.

Hawirko (1954) used the term "oil partition" to describe selective concentration of tubercle bacilli. He showed that if there were more than 10^5 tubercle bacilli in the mixture, some remained in the aqueous phase, If there were less than 10^5 organisms, all were taken up in the oil phase. The series of suspensions ranged from 10^9 down to 10^4 BCG bacilli per 10 ml of isotonic saline. The oils tested included castor, olive, cottonseed, peanut, corn, sesame, and paraffin.

Chapter III

PRELIMINARY INVESTIGATION

In the Literature Review we pointed out the long recognized factor of toxicity in established concentration procedures which employ strong acids or alkalies for digestion and decontamination. From the beginning we considered enzymes for this purpose. Sputum was the specimen of choice in all of these preliminary experiments. It is the most common specimen submitted for isolation of tubercle bacilli. Likewise sputum presents all the problems in itsprocessing that may be individual in other specimens. Inherently the problem is threefold (1) the selection of a digestant, (2) decontamination, (3) and concentration of the tubercle bacilli.

Enzyme Digestants

Experiment 1 **Selection of an Enzyme Digestant**

Materials: Pancreatin (Difco's Pangestin, 1:75); papain (Difco); pepsin (Difco, 1:10,000); trypsin (Difco, 1:250). Each enzyme was made up in 10% concentration.

N/1 NaOH, N/1 HCl, 0.04% brom thymol blue (BTB), 0.04% thymol blue, pooled sputum.

Procedure: Pooled sputum was thoroughly mixed with 4 mm glass beads in a Kahn shaker for 2 minutes. A 3 ml sample was added to each of 4 test tubes with 5 ml of water. The pH was adjusted to 7 with BTB in each specimen except the one for pepsin. The latter was adjusted to 1.5 with thymol blue indicator. One ml of each enzyme solution was added to each tube. The volume was brought to 10 ml with water.

Endpoint: The enzyme-sputum mixtures were observed at room temperature for 1 hour. The digestion was determined to be complete when an applicator stick could no longer pick up viscid strings of mucus. In negative results the mucus remained a viscid mass.

Table 1

SELECTION OF AN ENZYME

1% Enzyme	Disintegration of Mucus
pancreatin	+
papain	-
pepsin	+
trypsin	+
control (water)	-

Discussion: Time is a factor in enzyme activity. In this qualitative experiment the observation of each tube every few minutes at room temperature was continuous. The mucus threads had disintegrated into shreds within 15 to 20 minutes. From thereon the shreds continued to breakdown. Temperature also influences enzyme activity. According to West and Todd (1952) gastro-intestinal enzymes have optimum activity at 40 C, the peak on a bell shaped curve. Since 37 C incubation is available in every laboratory and the same temperature is optimum for growth of human tubercle bacilli, this was adopted as a norm for all subsequent experiments.

The concentration of the enzyme influences the rate of reaction in arithmetic progression. Until

further investigation, 1% final concentration was arbitrarily chosen.

At this time pancreatin was adopted for use. Pepsin was eliminated on the basis of the acid pH, and trypsin for economy, which later proved invalid.

During the investigations of the problem of decontamination it was found that the enzyme solution needed to be sterile to eliminate still another source of contamination.

Experiment 2. Activity of Sterilized Pancreatin

Materials: Pooled utum, 2% and 1% pancreatin

Procedure: Each dilution of pancreatin was treated as follows: 1) Seitz filtered, 2) autoclaved, and 3) the dry weight of pancreatin autoclaved before putting into solution. The sputum was mixed in a 1:1 ratio with each kind of sterilized enzyme solution. Unsterile enzyme solution served as control.

Results: Table 2

Table 2

ACTIVITY OF STERILIZED PANCREATIN

	Digestion Time	
Pancreatin	2%	1%
Untreated	10 min.	20 min.
Seitz filtered	no digestion	no digestion
Dry weight autoclaved	" "	" "
Solution autoclaved	" "	" "

Discussion: Pancreatin, or the commercial product pangestin, is a crude substance, and a 1% solution filtered with great difficulty. For thisreason, trypsin was used from hereon as an alternative. The greater activity of trypsin allowed a lower percentage solution which facilitated Seitz filtration. Autoclaving apparently denatured the protein so that it was not useful as an enzyme.

Experiment 3 Activity of Sterilized Trypsin

Materials: Pooled sputa, 1% trypsin

Procedure: 1:1 volume of sputa and trypsin, pH 7 with BTB.

Table 3

ACTIVITY OF STERILIZED TRYPSIN

Trypsin	Digestion Time	Temperature
untreated	20 min.	25 C
Seitz filtered	30 "	25 C
untreated	20 "	56 C

Discussion: Seitz filtration would insure a sterile solution which could easily be prepared in any laboratory. Ina concurrent experiment with the decontamination problem, contaminants were killed in the several trials made by heating the solution at 56 C for 10 minutes. Seitz filtering was preferred in spite of superficial advantages of heat decontamination. We felt thatthe contaminants would be variable from one sample to another. The slight loss in activity due to filtration is not significant. The 56 C would not kill ubiquitous spores.

Experiment 4 Activity of Trypsin at various pH levels

The Difco preperation of trypsin is described as being most effective at pH 7.8. Most fresh specimens of sputum ofthe hundreds checked in this laboratory were found to have an approximate pH of 7.

Materials: 10 specimens of sputum, Beckman pH meter, 1% trypsin, N/1 NaOH, N/1 HCl.

Procedure: Samples of trypsin were adjusted to pH 6.8, 7, 7.5, 8.0, 9.0.

Each trypsin solution was added to an equal volume of sputum.

Results: There was no apparent difference in 2 hours of of digestion at 37 C in this pH range.

Discussion: Since it is an established fact that tubercle bacilli grow best at pH 7, this was adopted also as the pH of the digesting agent in subsequent experiments with trypsin.

Experiment 5 Concentration of Trypsin Solution

Materials: Filtered trypsin solution ranging from 0.1 to 1.0%. One tenacious sputum specimen "cut" into six portions.

Procedure: Sputum and respective trypsin solutions were added in 1:1 ratio. The pH was adjusted to 7 with BTB.

Table 4

CONCENTRATION OF TRYPSIN

Trypsin	Digestion Time
0.1%	negative after 5 hours
0.2%	"
0.3%	"
0.4%	one hour
0.5%	"
1.0%	"

Discussion: The results indicate that 0.5% trypsin solution should be satisfactory for all types of sputa since most sputa are less tenacious than the sample used.

Many of the preliminary experiments to study the problem of contaminants and the third problem of concentration of tubercle bacilli were performed with the use of trypsin as a digestant. Trypsin digests the tenacious mass of sputum very well. But during these experiments it was also observed to digest the egg media such as Lowenstein-Jensen. Tubercle bacilli grew luxuriantly. Yellow-white colony masses could be fished from the remains of a slant, and a loopful from the hydrolyzed content yielded luxuriant growth on subculture.

This presented a practical problem. The egg media, Lowenstein-Jensen and Petragnani, are used almost universally. It would not be wise to develop a procedure that could not use egg media slants.

At this time the suggestion to use pepsin was a welcome solution to the problem. Pepsin (Difco) which digests casein 1:10,000 is active at an acid pH. Pepsin would no longer be active and would not attack the culture medium if the digestion would be neutralized to a pH 7 prior to inoculation.

Experiment 6 Activity of 0.5% Pepsin

Materials: 0.5% Seitz filtered pepsin solution, N/1 HCl, 0.04% thymol blue indicator, ten sputum specimens.

Procedure: The pH of the pepsin was adjusted to 1.5 with N/1 HCl. The sputa were mixed with pepsin in 1:1 ratio.

Results: Digestion of 10 specimens was complete in 20 minutes. Water controls adjusted to the same pH with HCl showed no digestion in the observed time.

Discussion: Activity of pepsin is comparable to that of trypsin. The acid control satisfied the question of digestion due to the acidity present.

In the literature gastric acidity is frequently shown to be toxic to tubercle bacilli. We have cited in the Literature Review Vincent and Birge (1947), Schwarting (1945, 1948), Kramer (1946), Vandiviere, Smith, and Sunkes(1952). For thisreason we did not accept HCl without question as the activating acid in pepsin digestion of sputum. Two organic acids, phosphoric and citric, were studied along with HCl. These acids in as low as 0.1 molar concentration were found to be good buffers when a 1:1 ratio with pepsin was made. Ten sputum specimens and each of the three acid-pepsin solutions retained their pH when mixed in a 1:1 ratio (Beckman pH meter). Acetic acid was also tried but appeared

unreliable in both steps and was dropped at this point.

Experiment 7 Toxicity of Components of Pepsin Digestant

Materials: 0.1M citric, 0.1M phosphoric, and 0.1M HCl acids. Pepsin (0.5%), Seitz filtered. H37Rv stock culture, Lowenstein-Jensen (L-J).

Procedure: A saline suspension of the stock H37Rv was prepared. Combinations of water and acids, acids and pepsin, pepsin and water were made up in equal volume of 4 ml each.

One tenth ml of culture suspension was added to each tube.

The tubes were incubated for 2 hours at 37 C.

The tubes were agitated and 0.1 ml from each was inoculated on 2 tubes of L-J medium. Growth was recorded at at 7, 14, 21 days.

Table 5

TOXICITY OF PEPSIN AND ACIDS

Growth after indicated days of incubation, 37 C

Acids:	Water + Acid			Pepsin + Acid		
Days:	7	14	21	7	14	21
Citric	3+	4+	4+	3+	4+	4+
Phosphoric	1+	2+	4+	1+	2+	4+
Hydrochloric	1+	2+	4+	1+	2+	4+
None	4+	4+	4+	4+	4+	4+

Discussion: It is apparent from table 5 that both phosphoric and hydrochloric acid are toxic to the initiation of growth of tubercle bacilli. Since 192 gm/liter of citric acid is molar, 0.1% would be equivalent to 2% in roundnumbers. This percentage was used from here on for further evaluation.

Experiment 8 Combinations of Pepsin and Citric Acid in the Digestion of Sputum

Materials: Citric acid in concentrations of 4%, 3%, 2%, and 1%. Filtered pepsin in concentrations of 1%, 0.6%, 0.5%, 0.1%. Three sputum specimens.

Procedure: The 4 strengths of pepsin and citric acid were made up in 1:1 volume in the 16 combinations to give final strengths represented on the table. A small clot of sputum from the pooled specimens was added to each. The tubes were allowed to incubate for 2 hours at 37 C. Digestion was indicated by a breakdown of the mucoid clot into a fine particulate suspension.

No digestion -- mucoid clot remained, could be lifted from tube with a wooden applicator stick.

Table 6

CITRIC ACID AND PEPSIN COMBINATIONS FOR OPTIMUM DIGESTION OF SPUTUM

	Citric Acid			
	0.5%	1.0%	1.5%	2.0%
Pepsin:				
0.1%	-	-	+/-	+
0.25%	+/-	+	+	+
0.3%	+	+	+	+
0.5%	+	+	+	+

Discussion: For convenience the digestant is made up in combination of 2% citric acid and 0.5% pepsin. This digestant is added in 1:1 ratio to sputum to give the final optimum concentration of 1% citric acid and 0.25% pepsin. In the many experiments performed before the final comparative study this proved reliable.

The Problem of Decontamination

Sputum may contain a variety of bacteria which can interfere with the isolation of tubercle bacilli. The problem of their destruction or removal from the sputum will be considered under the heading of decontamination.

In our preliminary investigations we experimented with several agents other than strong acids and alkalies. Some of these experiments overlap with digestion and concentration procedures, but they will be presented from the aspect of decontamination.

Experiment 9 Antibiotics in Decontamination

Materials: 1000 mg of aureomycin incorporated into 15 ml of nutrient agar, 1000 units of penicillin incorporated into 15 ml of melted nutrient agar. Ten sputum specimens.

Procedure: Each specimen was inoculated on a plate of each antibiotic medium and a control plate without antibiotic. Incubation for 24 hours at 37 C.

Results: Antibiotics showed no inhibition of contaminants relative to the controls.

Experiment 10 Antibiotic Discs in Decontamination

Materials: Impregnated discs -- aureomycin (30 mcg), bacitracin (10 units), chloromycetin (30 mcg), terramycin (30 mcg), penicillin 30 units). Nutrient agar plates and 10 sputum specimens.

Procedure: The plates were inoculated with the respective specimens and the discs placed with even spacing over the inoculum. Control plates were made without discs. Incubation for 24 hours at 37 C.

Results: No significant inhibition relative to the control plates.

Discussion: Antibiotics do not appear to be effective in the suppression of the heterogeneous flora that can be found in sputa.

Experiment 11 The Effects of 2 hour Trypsin Digestion and Drying on Contaminants

Materials: Blood agar plates with unglazed porcelain covers, sputum, 0.5% sterile trypsin.

Procedure: The specimen was divided, one part for control growth and Gram stain, 5 ml of the second part digested for 2 hours with an equal volume of 0.5% trypsin.

Two ml of each part were spread over each of

Procedure: continued

five blood agar plates.

The plates were incubated with unglazed porcelain covers "topside up" for 24 hours, then inverted for another 24 hours incubation.

Results: After 24 hours the liquid content was sufficiently reduced to allow the plates to be inverted.

Profuse growth of contaminants followed.

Experiment 12 Two Hour Trypsin Digestion, Drying, Penicillin, 70% Alcohol: Their Effect on Contaminants

Materials: Blood agar plates with unglazed porcelain covers, sputum, 0.5% sterile trypsin, penicillin, 70% alcohol.

Procedure: The specimen was divided, one part for control growth and Gram stain, a second portion was digested for 2 hours with an equal volume of 0.5% trypsin. After the 2 hour period the digested portion was divided, 30 000 units of penicillin were added to 2 ml, and 1 ml of 70% alcohol was added to another 2 ml of the digested sputum.

Five blood agar plates were generously streaked each with ½ ml of penicillin and digest, and 5 other plates were generously streaked each with ½ ml of the 70% alcohol and digested sputum.

One plate was inoculated with untreated sputum as a

Procedure: continued

control. Plates were incubated topside up, then inverted after 24 hours.

Results: There was no significant reduction of contaminants with either reagent.

Experiment 13 Effects of Complete Drying Before Inoculation

Materials: Pooled sputum, enzyme digested.

Penicillin (30,000/ml), 70% alcohol.

Petri dishes, unglazed porcelain tops, nutrient agar plates.

Procedure: Enzyme digested sputum was divided into 3 equal portions in petri dishes, covered with porous covers, incubated 24 hours with topside up. After 24 hours the dry, thin films were resuspended respectively in 2 ml of sterile water, 2 ml of 70% alcohol, 30 000 units of penicillin in 2 ml of water. Each suspension was poured on an agar plate, covered with a porous top, incubated overnight. They were incubated an additional 24 hours in an inverted position.

Results: 1) Direct inoculation of enzyme digest produced profuse growth of contaminants.

2) Enzyme digest dried and resuspended with water

Results: continued

showed about half as much contamination as (1).

3) Enzyme digest dried and resuspended with penicillin produced no growth for the first 24 hours, profuse growth after 48 hours.

4) Enzyme digest dried and resuspended with 70% alcohol remained negative after 48 hours incubation.

Discussion: We concluded that drying is a limited decontaminant in itself. The effect of drying on tubercle bacilli was a question for investigation. In repetition, seeded specimens of the water control from the preceding experiment appeared to yield very poor growth of tubercle bacilli in comparison to the controls in which the same size of inoculum was placed directly on the medium.

Experiment 14 <u>The Use of Glycerol to Eliminate Trauma to Tubercle Bacilli Due to Drying</u>

Materials: Trypsin digested sputum, glycerol, H37Rv suspension, L-J medium.

Procedure: A mixture of 3 ml of digested sputum, 1 ml of glycerol, 0.1 ml of tubercle bacilli suspension was placed into a petri dish, porcelain top. After overnight incubation, a viscid film remained which could be brushed onto 3 L-J tubes with a bent

Procedure: continued

glass rod. Controls consisted of the same size inoculum in 1 ml of water directly inoculated on L-J tubes.

Results: A loopful of the glycerol film inoculated on an agar plate grew no contaminants, nor did any grow on L-J medium.

Growth of tubercle bacilli was meager on the treated specimen in comparison to the control tubes. In this experiment we have recovery of the tubercle bacilli, although meager in growth, and elimination of contaminants.

Experiment 15 <u>The Toxicity of Alcohol for Tubercle Bacilli</u>

Materials: 70% alcohol, suspension of tubercle bacilli, L-J medium.

Procedure: Fourteen tubes with 1 ml of alcohol were made up to contain dilutions ranging from 70% down to 10% by volume. Each was seeded with a drop of suspension of tubercle bacilli. The entire ml, after 2 hours incubation at 37 C, was inoculated on 2 tubes of L-J medium.

Results: The first growth appeared in 7 days on the tubes which had been inoculated from the 24% alcohol

Results: continued

suspension or less. Scattered growth appeared on 3 tubes of higher concentrations of alcohol. The tubes were incubated for 21 days.

Discussion: (In General Discussion after experiment 17)

Experiment 16 Alcohol and Glycerol Toxicity on Tubercle Bacilli in Sputum

Materials: Pooled positive sputum, 0.5% trypsin, 10% Na_3PO_4, glycerol, 2% alcohol, L-J medium.

Procedure: The specimen was divided into 4 portions.

1) Control: Na_3PO_4 treatment, concentration by centrifugation.

2) Trypsin digested with 2 hr incubation, addition of 2 ml glycerol and 3 ml water.

3) Trypsin digested with 2 hr incubation, addition of 2 ml glycerol and 3 ml 2% alcohol.

4) Trypsin digested with 2 hour incubation, addition of 2 ml water and 3 ml 2% alcohol.

Portions 2, 3, and 4 were subjected to drying as previously described, resuspended, and inoculated on L-J medium.

Results: No growth appeared after 3 weeks of incubation in any tube except the 10% Na_3PO_4 treated specimen.

Discussion: (In General Discussion after experiment 17)

Experiment 17 Effects of Time on Exposure of Tubercle Bacilli to 2½% Alcohol

Materials: 2½% alcohol, H37Rv suspension, L-J medium.

Procedure: A suspension of organisms was made up in 2½% alcohol. One tenth ml was directly inoculated on tubes of L-J medium. This was repeated after 30, 45, and 60 minutes of exposure.

Results:

Table 7

EFFECT OF 2½% ALCOHOL ON VIABILITY OF TUBERCLE BACILLI

	Time in Alcohol Suspension			
	0	30 min.	45 min.	60 min.
Observed Growth after 3 days	+	-	-	-
6 "	2+	+	-	-
9 "	4+	2+	-	-
13 "	4+	2+	-	-
21 "	4+	4+	-	-

Discussion: (Experiments 15, 16, 17)

The drying procedure might have been a neat procedure to reduce the liquid content of an enzyme digested specimen of sputum, but it is not useful since it has a deleterious effect on the tubercle bacilli. The drying procedure was useful in ridding the specimens of a large portion of

contaminants.

Glycerol and alcohol were toxic for the tubercle bacilli.

Experiment 18 Sodium Azide, Potassium Tellurite, Zephiran: Effectiveness in Sputum Decontamination

Materials: 10 sputum specimens.

Nutrient agar pour plates with NaN_3 (1:5000) and K_2TeO_3 (1:100 000) individually and in combination.

Zephiran (1:2,000), steril swabs.

Procedure: NaN_3 was added to the agar before autoclaving.

K_2TeO_3 was Seitz filtered and added to melted agar aseptically.

The swabs were thoroughly stirred in each specimen of sputum and then placed in a tube of zephiran for 2 hours at room temperature.

Each swab was inoculated on each kind of plate and a control plate that had no added chemical.

Swabs without zephiran treatment were inoculated on each plate with and without chemical.

Results: Table 8

Table 8

GROWTH OF SPUTUM CONTAMINANTS WITH NaN_3 and K_2TeO_3 IN COMBINATION WITH ZEPHIRAN

Agar Pour Plates	NaN_3		K_2TeO_3		NaN_3+K_2TeO_3		control	
Zephiran	no	yes	no	yes	no	yes	no	yes
Sputum								
1	-	-	+	-	-	-	+	-
2	-	-	+	-	-	-	+	-
3	-	-	+	-	-	-	+	-
4	-	-	+	-	-	-	+	-
5	+	-	+	-	-	-	+	-
6	+	-	+	-	-	-	+	-
7	-	-	+	-	-	-	+	-
8	-	-	+	-	-	-	+	-
9	-	-	+	-	-	-	+	-
10	-	-	+	-	-	-	+	-

Discussion: The experiment shows the effectiveness of zephiran treatment to destroy contaminants. In these ten specimens NaN_3 and K_2TeO_3 in combination were effective without zephiran. From the first column thiswould appear to be due to NaN_3.

Experiment 19 Zephiran-Trypsin Digested Sputum

Materials: Ten sputum specimens, 1% trypsin (sterile); zephiran in 1:1000, 1:10 000 dilutions; nutrient agar plates.

Procedure: The 10 specimens were digested for 2 hours in a digestant containing in combination 0.5% trypsin and zephiran. The zephiran was varied in concentration. Agar plates were heavily inoculated.

Table 9

SPUTUM CONTAMINANT GROWTH AFTER ZEPHIRAN-TRYPSIN

	Zephiran		
	1:2000	1:5000	1:10 000
Sputum			
1	-	-	-
2	-	-	-
3	+	+	+
4	+	+	+
5	-	-	-
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-

Discussion: In specimens 3 and 4 the contaminant that

persisted was Pseudomonas sp. Decontamination by the use of of zephiran was discontinued at this time. With the substitution of pepsin for trypsin, zephiran was unnecessary.

Experiment 20 Plant Hormones as Contaminant Inhibitors

At one period in the investigation we were working with plant hormones as growth factors for M. tuberculosis. In high concentration they are known to be toxic to plants, however in low concentration they may stimulate bacterial growth as they do in the plants. Simultaneously with tubercle bacilli, E.coli, beta hemolytic streptococci, and Candida albicans were appraised. Inhibition of growth was more apparent than stimulation in the dilutions tested.

Materials: Basic agar (for tubercle bacilli)

Cultures: M. tuberculosis, E. coli, beta Streptococcus, C. albicans.

Plant hormones (indicated in data by number)

1. 3.7% a-naptholene acetic acid (M/5 NaOH)
2. 5.0% o-iodobenzoic acid (M/5 NaOH)
3. 2.7% phenyl acetic acid (M/5 NaOH)
4. 3.7% o-chlorophenoxy acetic acid (M/5 NaOH)
5. 4.4% 2,4 dichlorophenoxy acetic (M/5 NaOH)
6. 3.7% iodoacetic acid (M/5 NaOH)
7. 3.6% paranitrophenyl acetic (M/5 NaOH)

8. 5.0% paranitrophenyl acetic (M/5 NaOH)

9. 3.0% tri-iodobenzoic acid (M/15 NaOH)

10. 3.5% indol-3-acetic acid (M/5 NaOH)

Procedure: Basic agar slants of 5 ml each were prepared. Hormone dilutions were made so that with the addition of 1 ml of the hormone to the agar slant final concentrations of the hormone would be 0.5%, 0.1%, 0.025%, 0.005%, 0.001%, 0.00024% respectively in each set of tubes. The tubes were incubated 3 days to allow diffusion of the hormone into the agar and partial evaporation of surplus moisture. This also insured sterility of the hormone-agar media. Cultures were examined after 24, 48, 72 hours, and for 3 weeks for the tubercle bacilli.

Table 10

GROWTH OF BACTERIA ON HORMONE AGAR

	Hormone Concentration in Agar						
	0.5%	0.1%	0.25%	0.5%	0.1%	0.25%	0.005%
	E. coli			b-Streptococcus			
Hormone							
1	+	+	+	-	+	+	+
2	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+
6	-	+	+	-	-	-	+
7	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+
9	-	-	+	+	+	+	+
10	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+

Discussion: In the hormone-agar experiment we have included only that which gave positive results from the aspect of inhibition of growth. There was no inhibition of C. albicans. Various yeasts are common contaminants of sputum. Hormone #6 or iodoacetic acid showed some promise in a 0.5% concentration. It did not have a sufficiently wide range as whown by inoculation of a digested sputum.

These experiments do not solve the problem of ridding a sputum specimen of contamination. The solution of the problem appeared with the use of the enzyme pepsin and the process of concentration of tubercle bacilli. The experimental evidence will be presented in the following pages.

Concentration of Mycobacterium tuberculosis

In the past centrifugation has been used as the most common method of concentration of tubercle bacilli. Another method cited in the Literature Review consisted of layering the tubercle bacilli in an interface of water and hydrocarbon. We have found the latter method most efficient since it gave selective concentration of tubercle bacilli. This we worked out experimentally before happening upon the papers by Mudd and Mudd (1924) and Reed and Rice (1931) which treat the subject comprehensively.

Experiment 21 Selection of a Concentration Agent

Materials: Suspension of tubercle bacilli (H37Rv).
Eighteen chemical agents selected on the basis of low boiling point and low specific gravity. Several were included simply because they were available from the shelf. See table 11.

Procedure: Eighteen tubes with 2 ml of water containing 0.02 ml of bacterial suspension were lined up. One ml of an agent was added to each tube and thoroughly agitated with pipette and bulb. (Oral pipetting was strictly avoided throughout this work. A 3 ml rubber bulb on a 5 ml pipette

Procedure: continued

was effective and safe to agitate a suspension of tubercle bacilli in water and agent mixture.) The tubes were allowed to stand 1 hour. A fine white film was macroscopically visible at the interface in many tubes.

Slides were marked with a wax pencil area of a square cm. A Kahn pipette and small bulb were used to transfer 0.05 ml of concentrate to slide and each of 2 tubes of L-J medium. Controls for acid fast stain and culture were included.

Key to Table II:

* Degree of clarity of water and concentrating agent.

4+ indicates a white film of organisms at the interface with clear water and agent phases.

+ indicates a film at the interface with a very fine suspension of particles in either phase.

- no apparent concentration

** Degree of intace morphology and stain reaction

- complete distortion of bacilli

*** The numbers indicate a count of the colonies

Dense -- a thick blanket of colonies

- no growth after 4 weeks.

**** Control -- 0.02 ml of inoculum used directly

Table 11

EFFECTS OF CONCENTRATING AGENTS ON TUBERCLE BACILLI

Agent	Macroscopic Appearance*	AFS**	Culture 4 wks Growth***
N/1 amyl alcohol	4+	+	-
iso amyl alcohol	4+	4+	-
N/1 butyl acetate	4+	4+	-
toluene	4+	2+	-
hexone	4+	4+	3, abnormal
carbon tetrachloride	4+	4+	-
monochlorobenzene	+	4+	6, abnormal
2-chloro-2-methyl benzene	+	4+	-
isoprene	+	4+	2, normal
diacetyl	-	-	-
methyl formate	-	-	-
methylal	4+	4+	-
3-chloropropene	4+	4+	-
propylene oxide	-	-	-
n-pentane	4+	4+	dense, normal
para cymene	4+	4+	50, luxuriant
alpha terpeneol	4+	4+	-
terpinolene	4+	4+	dense, normal
None (control)****		4+	dense, normal

Experiment 22 Toxicity of Pentane and Terpinolene

Materials: Dubos broth culture of 2 weeks growth (H37Rv). Terpinolene, pentane, 6 tubes of 2 ml water.

Procedure: A loopful of culture was added to each of the 6 tubes.

Terpinolene was added to 3 tubes, and pentane to 3 tubes (1 ml each).

The contents of the three sets of tubes were thoroughly agitated with a pipette.

L-J medium was inoculated after exposure time of 5, 30, 150 minutes. Controls were inoculated with 1 loopful of broth culture.

Table 12

TOXICITY OF PENTANE AND TERPINOLENE

	Culture Growth		
	Pentane	Terpinolene	Control
Exposure:			
5 min.	4+	4+	4+
30 "	4+	4+	4+
150 "	4+	1+	4+

Discussion: Terpinolene shows toxicity with a 2½ hour exposure time. It is likewise not as easy to handle with its oily consistency as pentane. The latter has a boiling point of 36.3 C and a specific gravity of 0.63. Its volatility is an advantage

Discussion: continued

since the pentane that is carried over into the culture tube or to the slide evaporates quickly. We used the Matheson, Coleman, & Bell product.

To supplement the above experiment we put a loopful of Dubos broth culture into a tube of ½ ml of pentane. After 2½ hours the entire content was transferred to L-J medium. After 24 hours incubation the pentane had evaporated from the slant. The colonies grew as luxuriantly as in a control tube which was inoculated directly with a loopful of the broth culture.

Experiment 23 <u>Sodium Chloride as a Flotation Agent</u>

Materials: NaCl in 1% to 5% concentrations, Dubos broth culture of 2 weeks growth.

Procedure: Two hundredths drop of culture was well agitated in 3 ml of each concentration of NaCl. The tubes were allowed to stand ½ hour. Two tubes of L-J medium were each inoculated with a loopful of the floating film from the respective concentrations of NaCl. The cultures were incubated for 2 weeks.

Table 13

EFFECT OF NaCl USED AS A FLOTATION AGENT

Concentration	Growth
5% NaCl	4+
4% "	2+
3% "	2+
2% "	1+
1% "	-
water	1+
direct inoculation	4+

Discussion: If flotation would be considered as a concentration process, the effect on contaminating organisms must also be accounted for. Another experiment was planned to repeat the above with other organisms and a comparison made with pentane and terpinolene.

Experiment 24 NaCl Flotation and Interface Concentration of Representative Bacteria

Materials: NaCl in 1% to 5% concentrations.

Pentane and terpinolene, cultures of Streptococcus, E. coli, C. albicans.

Procedures: Suspensions of the cultures were prepared.

A tube of 3 ml of each NaCl solution was inoculated

Procedure: continued

with 0.02 ml of each culture. The same was inoculated into 3 ml of water for the pentane and terpinolene. All tubes were well agitated. After ½ hour, blood agar plates were inoculated and incubated for 24 hours.

Table 14

EFFECT OF NaCl, PENTANE, AND TERPINOLENE ON SOME BACTERIA

	Growth of Bacteria		
Agent:	Streptococcus	E. coli	C. albicans
5% NaCl	1+	4+	1+
4% "	3+	4+	1+
3% "	4+	4+	1+
2% "	4+	4+	4+
1% "	4+	4+	4+
pentane	1+	4+	1+
terpinolene	1+	-	-

Discussion: From experiments 23 and 24 it would appear that 5% NaCl is equal to pentane as a selective concentrating agent of tubercle bacilli. Supplementary to these findings we determined how toxic 5% NaCl would be for tubercle bacilli that had been exposed to 5% NaCl for 2½ hours. Control

Discussion: continued

inocula grew normally and cultures of NaCl exposed inocula were negative. Pentane exposure for 2½ hours did not effect the viability of tubercle bacilli. Pentane is also lethal to many contaminants. Swabs were well agitated in 15 sputum specimens and then allowed to stand in tubes of pentane for ½ hour. Blood plates were inoculated with untreated swabs and pentane treated swabs. The results were variable, but in all cases there was a noticeable reduction of contaminant growth from the pentane treated inocula.

Experiment 25 Effectiveness of Pentane Concentration

Materials: Enzyme digested positive sputum, pentane

1) 25 ml burette
2) glass tubing (150 x 15 mm) with rubber cap diaphrem at one end; i.e., tube with rubber bottom, 2 ml syringe, 20 gauge needle.
3) test tube (150 x 15 mm)

Procedure:

1) Burette: 10 ml of digest were well agitated with 1 ml of pentane, poured into theburette and allowed to stand ½ hour.

Procedure: continued

The lower 1/3 was allowed to empty into a receiving tube, the middle 1/3 into a second tube, the top 1/3 (immediately under the interface) into a third tube, the interface and pentane into a fourth tube. Each receiving tube was examined for acid fast bacilli and contaminants. (by stain)

2) The glass tubing with the rubber cap diaphram bottom served as a tube. Ten ml of digest was well agitated with 1 ml of pentane, poured into the tube and allowed to stand ½ hour. With a syringe and needle ¼ portions were removed into receiving tubes by pushing the needle through the diaphram, in the same manner as the stopcock served in the burette.

3) Frozen tube: 10 ml of digest were well agitated with 1 ml of pentane, poured into an ordinary 150 x 15 mm tube and allowed to stand ½ hour. The tube and contents were quickly frozen by exposure to - 20 C.

The frozen contents were removed from the tube by briefly immersing it into hot water. The frozen "plug" was divided into four portions comparable to methods (1) and (2).

Table 15

EFFICIENCY OF PENTANE CONCENTRATION

			Inter-face	Top 1/3	Mid 1/3	lower 1/3
1)	Burette	A-F Bacilli	many	-	-	-
		Contaminants	few	many	many	many
2)	Tubing	A-F Bacilli	many	-	-	-
		Contaminants	few	many	many	many
3)	Frozen Tube	A-F Bacilli	many	-	-	-
		Contaminants	few	many	many	many

Discussion: Our findings appear to be similar to Reed and Rice (1931). They found that with most hydrocarbons Mycobacteria sp. would concentrate to the extent of about 95% in the non-aqueous phase. Other organisms (non-acid fast) concentrated in the aqueous phase.

Chapter IV

THE PEPSIN-PENTANE CONCENTRATION PROCEDURE

Materials:

1) Digestant -- 0.5% pepsin and 2% citric acid in distilled water, Seitz filtered.

We used the Difco product which gives the proteolytic titer of 1:10,000 for casein. The citric acid was of reagent grade. We found it best to prepare the solution and allow it to stand several hours or overnight before filtering. After this period the solution is almost clear and there is negligible loss of pepsin activity with Seitz filtration.

2) Pentane -- This simple five carbon chain is highly volatile with boiling point at 36 C and specific gravity of 0.63. (Matheson, Coleman & Bell) We found a bunsen burner flame unnecessary throughout the procedure which eliminates the fire hazard.

3) Digestion tube -- This is the ordinary 75 ml culture tube which resembles the NPN tube commonly used in biochemistry. An NPN tube support is also useful. We found cotton plugs a dangerous nuisance. To cover the digestion tube, small glass beakers or aluminum caps are much more efficient.

4) 3 ml rubber bulb and 5 ml pipette -- We rigidly

avoided oral pipetting throughout the study. The use of a bulb with a pipette is a simple and safe technique when dealing with pathogens. The 3 ml bulb on the 5 ml pipette permits a safe margin. As a receptacle for the pipette when not in use, an empty 5 inch culture tube along side the digestion tube is handy.

50 ml compression bulb and 12 inch piece of rubber tubing -- In processing a number of specimens the agitation of the pentane mixture becomes tiresome. We devised the compression bulb-tubing assembly for the pipette in place of the 3 ml bulb. The hand that holds the bulb can rest on the table top. The bulb-tubing assembly is attached to the pipette, and the mixture is rapidly and efficiently agitated. An alternate method is with an air jet in those laboratories where it is available. The jet is connected to the pipette with a length of rubber tubing. A clamp on the tubing is necessary for proper control.

5) N/1 KOH -- This was found to be a better neutralizing alkali than N/1 NaOH which was initially used. With the NaOH a frothy layer formed in the pentane phase. Time is wasted in waiting for it to condense to a thin flat layer at the interface. The pH is adjusted to a faint green with BTB indicator.

Procedure:

1) Add an equal volume of digestant to the specimen. This need not be measured accurately. Sputa vary in consistency. A 1:1 ratio was found to take care of the most tenacious, but it may be in excess of the needs of many specimens. Visual approximation of the amount that would double the volume of the contents of the container is sufficient. The specimens which are quite watery or have little mucus could be processed in a shorter time rather than to reduce the amount or proportion of the digestant.

2) After 2 hours of incubation at 37 C, transfer mixture to digestion tube. Specimens sometimes give the appearance of not being completely digested. The larger shreds which have failed to break down easily will frequently do so by forced aspiration with a 5 ml pipette and 3 ml bulb. In the comparative study those specimens that were stubbornly tenacious after 2 hours were likewise so with the 10% Na_3PO_4 procedure at that period. An additional hour was usually sufficient incubation. If the greater part of the specimen is digested, the processing should not be delayed for a few stubborn shreds. With the necessary agitation in mixing the pentane-digestion mixture, the tubercle bacilli have a good chance to be freed from mucus threads. The acidity is neutralized to a faint green (BTB) with N/1 KOH.

3) Add 3 or 4 ml of pentane (about an inch of pentane over the surface of the digest). The digestion tube should not be more than one half full, otherwise vigorous agitation with the pentane is not possible. The compression bulb, tubing, and pipette assembly is the most efficient. A little practice with a deeply stained water and pentane will show how effectively the aqueous phase is forced through the pentane phase. The objective is to expose the bacilli to the pentane. Allow the agitated mixture to stand 10 or 15 minutes. The tubercle bacilli are concentrated at the interface of aqueous and pentane layers.

4) Aspirate the interface for slide and culture. The pentane need not be removed to aspirate the interface. The little carried along in the inoculum will not matter since it ishighly volatile. The same pipette can be used throughout the procedure for one specimen. At least 2 tubes of medium should be inoculated, and 3 or 4 tubes are preferable.

The Pepsin-Pentane Procedure

(in brief)

Materials: 1) Digestant -- 0.5% pepsin in 2% citric acid, Seitz filtered.

2) Pentane

3) Digestion tube (75 ml culture tube), 5 ml pipette, 3 ml rubber bulb, N/1 KOH, 0.04% brom thymol blue (BTB).

Procedure: 1) Add an equal volume of digestant to the specimen, incubate for 2 hours at 37 C.

2) Neutralise acidity with KOH to faint green (BTB)

3) Add 3-4 ml of pentane (an inch layer). Agitate the mixture thoroughly with bulb and pipette. No flame should be nearby. Pentane is very volatile.

4) Aspirate interface for slide and culture.

Chapter V

COMPARATIVE STUDY OF PEPSIN-PENTANE CONCENTRATION OF SPUTUM AND 10% Na_3PO_4-CENTRIFUGATION

Materials: Digestants -- 0.5% pepsin in 2% citric acid, 10% Na_3PO_4

Pentane, N/1 KOH, Lowenstein-Jensen (L-J) medium, brom thymol blue(BTB) indicator, 75 ml and 15 ml tubes, 5 ml pipettes, rubber bulb, Sputum specimens from local tuberculosis hospitals.

Procedure:

1) Each specimen was divided into two parts as nearly as possible. Alternate pipetting of 1-2 ml was the usual procedure, but some tenacious specimens had to be "cut" with scissors to divide them. The portions were placed in 75 ml tubes and an equal volume of pepsin digestant was added to one and 10% Na_3PO_4 to the other.

2) A few drops of BTB was added to each tube. The contents were thoroughly agitated. Distribution of color reaction helped to determine the endpoint of sufficient agitation. The tubes were incubated for 2 hours which was sufficient for digestion of most specimens.

Procedure: continued

3) If a digestion exceeded 10 ml in amount, only 10 ml of it were used for centrifugation and pentane concentration. The Na_3PO_4 digest was transferred to a 15 ml tube and centrifuged for 15 minutes at approximately 2000 rpm in an angle centrifuge. The acidity of the pepsin digest was neutralized with N/1 KOH to a faint green then agitated very thoroughly with an inch layer of pentane. The tubes stood at least 15 minutes to allow maximum concentration at the interface layer.

4) From each, centrifuged sediment and interface layer, 0.1 ml was inoculated on 2 tubes of L-J medium and incubated for 5 weeks.

5) From each concentration 0.05 ml wasused for the acid fast stain. A pattern used under the slide helped to mark off two areas of 2 cm x 1 cm with a red wax pencil. This allowed simultaneous staining from the two digestions of each specimen.

Table 16

DATA OF COMPARATIVE STUDY

	AFS		Cultures							
			Days: 14		21		28		35	
	T*	P*	T	P	T	P	T	P	T	P
Spec.										
1	-	+	-	+	-	+	-	+	-	+
2	+	-	+	-	+	-	+	-	+	-
3	-	-	-	+	-	+	-	+	-	+
4	+	+	+	+	+	+	+	+	+	+
5	+	+	-	+	-	+	+	+	+	+
6	-	+	-	+	-	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+
8	-	-	-	-	-	-	-	-	-	-
9	+	+	-	-	-	-	-	-	+	+
10	-	+	-	-	-	-	-	-	-	-
11	+	+	+	+	+	+	+	+	+	+
12	+	+	-	-	-	-	-	-	-	-
13	+	+	+	+	+	+	+	+	+	+
14	+	+	-	-	-	-	-	-	+	-
15	+	+	-	-	-	-	-	-	-	-
16	+	+	-	-	+	+	+	+	+	+
17	-	+	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-

\+ = pos.
\- = neg.

*T = 10% Na_3PO_4 procedure
*P = pepsin-pentane procedure

DATA OF COMPARATIVE STUDY

	AFS		Cultures							
			Days: 14		21		28		35	
	T*	P*	T	P	T	P	T	P	T	P
Spec. 19	+	+	-	-	+	+	+	+	+	+
20	--	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-
22	-	+	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-
25	-	+	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	+	-
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-
31	+	+	-	-	-	-	-	-	+	+
32	+	-	-	-	-	-	-	-	-	-
33	+	+	+	+	+	+	+	+	+	+
34	-	+	+	+	+	+	+	+	+	+
35	-	-	-	-	-	-	-	-	-	-
36	--	-	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-	-	-

*T = 10% Na_3PO_4

*P = pepsin-pentane procedure

DATA OF COMPARATIVE STUDY

	AFS		Cultures Days: 14		21		28		35	
	T*	P*	T	P	T	P	T	P	T	P
Spec. 38	-	-	-	-	-	-	-	-	-	-
39	-	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-	-
41	-	-	-	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-	-	-	-
43	-	-	-	-	-	-	-	-	-	-
44	-	-	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-	-	-
46	-	-	-	-	-	-	-	-	-	-
47	+	+	+	+	+	+	+	+	+	+
48	+	+	+	+	+	+	+	+	+	+
49	+	+	+	+	+	+	+	+	+	+
50	-	-	-	+	+	+	+	+	+	+
51	-	-	-	-	-	-	-	+	-	+
52	+	+	-	+	+	+	+	+	+	+
53	+	+	-	+	-	+	-	+	-	+
54	+	+	-	-	-	+	-	+	-	+
55	-	-	-	-	-	+	-	+	-	+
56	-	-	-	-	-	-	-	-	-	-

*T = 10% Na_3PO_4 procedure

*P = pepsin-pentane procedure

DATA OF COMPARATIVE STUDY

	AFS		Cultures							
			Days: 14		21		28		35	
	T*	P*	T	P	T	P	T	P	T	P
Spec. 57	+	-	-	-	+	+	+	+	+	+
58	+	-	-	-	-	-	-	+	-	+
59	+	-	-	-	-	+	-	+	-	+
60	+	+	-	-	+	+	+	+	+	+
61	+	+	-	-	+	+	+	+	+	+
62	+	+	-	-	+	+	+	+	+	+
63	-	+	-	-	-	-	-	-	+	+
64	-	+	-	-	-	-	-	-	+	-
65	-	+	-	-	-	-	-	-	-	-
66	-	+	-	-	-	-	-	-	-	-
67	-	+	-	-	+	-	+	-	+	-
68	+	+	-	-	+	+	+	+	+	+
69	+	+	-	-	-	-	-	-	-	-
70	+	+	-	-	-	-	-	-	+	+
71	+	+	-	-	-	-	+	-	+	-
72	+	+	-	-	-	-	-	-	+	+
73	+	+	-	-	-	-	+	-	+	-
74	-	+	-	-	-	-	-	-	+	+
75	+	+	-	-	-	+	-	+	-	+

*T = 10% Na_3PO_4 procedure

*P = pepsin-pentane procedure

DATA OF COMPARATIVE STUDY

	AFS		Cultures							
			Days: 14		21		28		35	
Spec.	T*	P*	T	P	T	P	T	P	T	P
76	+	+	-	-	+	+	+	+	+	+
77	-	-	-	-	-	-	-	-	-	-
78	-	-	-	-	-	-	-	-	+	-
79	-	+	-	-	-	-	-	-	-	-
80	-	+	-	-	-	-	-	-	-	-
81	-	+	-	-	-	-	-	-	-	-
82	-	+	-	-	-	-	-	-	-	-
83	-	+	-	-	-	-	-	-	-	-
84	-	+	-	+	-	+	-	+	-	+
85	+	+	-	-	+	+	+	+	+	+
86	+	+	-	+	-	+	-	+	+	+
87	-	-	-	-	-	-	-	-	-	-
88	-	-	-	-	-	-	-	-	-	-
89	+	+	-	-	-	-	-	-	-	+
90	+	+	-	-	-	-	-	-	-	+
91	-	-	-	-	-	-	-	-	-	-
92	-	-	-	-	-	-	-	-	-	-
93	+	+	+	+	+	+	+	+	+	+

*T = 10% Na_3PO_4 procedure

*P = pepsin-pentane procedure

DATA OF COMPARATIVE STUDY

	AFS		Cultures							
			Days: 14		21		28		35	
	T*	P*	T	P	T	P	T	P	T	P
Spec. 94	+	+	+	+	+	+	+	+	+	+
95	-	+	+	+	+	+	+	+	+	+
96	+	+	-	+	-	+	-	+	-	+
97	-	-	-	-	-	-	-	-	-	-
98	-	-	-	-	-	-	-	-	-	-
99	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-
101	-	-	-	-	-	-	-	-	-	-
102	-	-	-	-	-	-	-	-	-	-
103	-	-	+	+	+	+	+	+	+	+
104	-	-	+	+	+	+	+	+	+	+
105	-	+	+	+	+	+	+	+	+	+
106	+	+	+	+	+	+	+	+	+	+
107	+	+	-	-	-	-	-	+	-	+
108	+	+	-	-	-	-	-	-	-	-
109	-	+	+	+	+	+	+	+	+	+
110	+	+	+	+	+	+	+	+	+	+
111	-	-	-	-	-	-	-	-	+	+

*T = 10% Na_3PO_4 procedure

*P = pepsin-pentane procedure

DATA OF COMPARATIVE STUDY

	AFS		Cultures Days: 14		21		28		35	
	T*	P*	T	P	T	P	T	P	T	P
Spec. 112	-	-	-	-	-	-	-	-	-	-
113	-	-	-	-	-	-	-	-	-	-
114	-	-	-	-	-	-	-	-	-	-
115	-	+	-	-	-	-	-	-	-	-
116	-	+	-	-	+	-	+	-	+	-
117	-	-	-	-	-	-	-	-	-	-
118	-	-	-	-	-	-	-	-	-	-
119	-	-	-	-	-	-	-	-	-	-
120	+	+	+	+	+	+	+	+	+	+
121	+	+	-	+	+	+	+	+	+	+
122	-	-	-	-	-	-	-	-	-	-
123	-	-	-	-	-	-	-	-	-	-
124	-	+	-	-	-	-	-	-	-	-
125	-	-	-	-	-	-	-	-	-	-
126	-	+	-	-	-	-	-	-	-	-
127	-	-	-	-	-	-	-	-	-	-
128	+	+	-	-	-	-	-	-	-	-
129	-	-	-	-	-	-	-	-	-	-

*T = 10% Na_3PO_4 ppocedure

*P = pepsin-pentane procedure

DATA OF COMPARATIVE STUDY

	AFS		Cultures Days: 14		21		28		35	
	T*	P*	T	P	T	P	T	P	T	P
Spec. 130	-	+	-	-	-	-	-	-	-	-
131	+	+	-	-	-	-	-	-	-	-
132	+	+	-	-	-	-	-	-	-	-
133	-	+	+	+	+	+	+	+	+	+
134	+	+	+	+	+	+	+	+	+	+
135	-	-	-	-	-	-	-	-	-	-
136	-	-	-	-	-	-	-	-	-	-
137	-	-	-	-	-	-	-	-	-	-
138	+	+	-	+	-	+	-	+	-	+
139	+	+	-	-	-	-	-	-	-	-
140	-	+	-	-	-	-	-	-	-	-
141	+	+	-	-	-	-	+	+	+	+
142	-	-	+	-	+	-	+	-	+	-
143	+	+	+	+	+	+	+	+	+	+
144	-	-	-	+	-	+	-	+	-	+
145	-	-	-	+	-	+	-	+	-	+
146	-	-	+	+	+	+	+	+	+	+
147	+	-	+	+	+	+	+	+	+	+

*T = 10% Na_3PO_4 procedure

*P = pepsin-pentane procedure

DATA OF COMPARATIVE STUDY

	AFS		Cultures							
			Days 14		21		28		35	
Spec.	T*	P*	T	P	T	P	T	P	T	P
148	-	-	-	-	-	-	-	-	-	-
149	-	-	-	-	-	-	-	-	-	-
150	-	-	-	-	-	-	-	-	-	-
151	-	-	-	-	-	-	-	-	-	-
152	-	-	+	+	+	+	+	+	+	+
153	-	-	-	+	-	+	-	+	-	+
154	-	-	-	-	-	-	-	-	-	-
155	-	+	+	+	+	+	+	+	+	+
156	-	+	+	+	+	+	+	+	+	+
157	-	-	+	+	+	+	+	+	+	+
158	-	-	-	-	-	+	-	+	-	+
159	-	-	-	-	-	+	-	+	-	+
160	-	-	-	-	-	-	-	-	-	-
161	+	+	+	+	+	+	+	+	+	+
162	+	+	+	+	+	+	+	+	+	+
163	+	+	+	-	+	+	+	+	+	+
164	+	+	-	+	-	+	-	+	-	+
165	+	+	-	-	-	-	-	-	-	-

*T = 10% Na_3PO_4 procedure

*P = pepsin-pentane procedure

DATA OF COMPARATIVE STUDY

	AFS		Cultures							
			Days 14		21		28		35	
Spec.	T*	P*	T	P	T	P	T	P	T	P
166	-	-	-	-	-	-	-	-	-	-
167	+	+	-	-	-	-	-	-	-	-
168	-	-	-	-	-	-	-	-	-	-
169	-	-	-	-	-	-	-	-	-	-
170	-	+	-	-	-	-	-	-	-	-
171	+	+	-	-	-	-	-	-	-	-
172	-	-	-	-	-	-	-	-	-	-
173	-	-	-	-	-	-	-	-	-	-
174	-	-	-	-	-	-	-	-	-	-
175	-	-	-	-	-	-	-	-	-	-
176	-	-	-	-	-	-	-	-	-	-
177	-	-	-	-	-	-	-	-	-	-
178	-	-	-	-	-	-	-	-	-	-
179	-	-	-	+	-	+	-	+	-	+
180	+	+	-	+	-	+	+	+	+	+
181	-	+	-	-	-	-	+	-	+	-
182	-	-	-	-	-	-	-	-	-	-
183	+	-	+	+	+	+	+	+	+	+
184	+	-	+	-	+	-	+	+	+	+

*T = 10% Na_3PO_4 procedure

*P = pepsin-pentane procedure

DATA OF COMPARATIVE STUDY

	AFS		Cultures Days 14		21		28		35	
	T*	P*	T	P	T	P	T	P	T	P
Spec. 185	+	+	-	+	-	+	+	+	+	+
186	-	+	-	+	-	+	+	+	+	+
187	-	+	-	+	-	+	-	+	-	+
188	-	+	-	+	-	+	-	+	-	+
189	+	+	+	+	+	+	+	+	+	+
190	+	+	+	+	+	+	+	+	+	+
191	+	+	+	+	+	+	+	+	+	+
192	-	-	-	-	-	+	+	+	+	+
193	+	-	-	-	+	+	+	+	+	+
194	-	-	-	-	+	-	+	-	+	+
195	+	+	-	-	+	+	+	+	+	+
196	+	+	-	-	+	+	+	+	+	+
197	+	+	-	-	+	+	+	+	+	+
198	-	-	-	-	-	-	-	-	-	-
199	-	-	-	-	-	-	-	-	-	-
200	-	-	-	-	-	-	-	-	-	-
201	+	+	+	+	+	+	+	+	+	+
202	+	+	+	+	+	+	+	+	+	+

*T = 10% Na_3PO_4 procedure

*P = pepsin-pentane procedure

DATA OF COMPARATIVE STUDY

	AFS		Cultures Days 14		21		28		35	
	To	Po	T	P	T	P	T	P	T	P
Spec. 203	+	+	+	+	+	+	+	+	+	+
204	+	+	-	+	-	+	-	+	-	+
205	-	-	-	-	-	-	-	-	-	-
206	-	-	-	-	-	-	-	-	-	-
207	-	-	-	-	-	-	-	-	-	-
208	-	-	-	-	-	-	-	-	-	-
209	+	+	-	+	-	+	-	+	-	+
210	+	+	+	+	+	+	+	+	+	+
211	+	+	+	+	+	+	+	+	+	+
212	+	+	+	+	+	+	+	+	+	+
Total Positive	83	110	44	64	63	85	73	90	85	100

*T = 10% Na_3PO_4 procedure

*P = pepsin-pentane procedure

Discussion and Analysis of Results of Comparative Study

In diagnostic work we are interested not only in more reliable results but also early detection of colony growth. The tabulated data appears to show both. Table 17 was summarized from the data. The vertical columns indicate the number of specimens positive only by the 10% Na_3PO_4 method, the number positive only by the pepsin-pentane procedure, and those positive by both procedures. The total columns are sums of columns 1 and 3, 2 and 3 respectively. The percent columns are computed from the total columns; i.e., 44/68 = 65% and 64/68 = 94%.

Table 17

SUMMARY OF COMPARATIVE STUDY

	Positive Cultures							
Days	Only T*	Only P*	T & P	Total	Total T	Total P	T%	P%
14	4	24	40	68	44	64	65%	94%
21	6	28	57	91	63	85	69%	93%
28	8	25	65	98	73	90	74%	92%
35	11	26	74	111	85	100	76%	90%

*T = 10% Na_3PO_4 procedure

*P = pepsin-pentane procedure

The standard error of the difference of two proportions and the "t" test were applied to determine the significance of the percents in the last columns.

On the 14th day:

$$SE_d = \sqrt{\frac{65 \times 35}{68} + \frac{94 \times 6}{68}} = 6.5 \qquad 94\% - 65\% = 29\% \text{ observed difference}$$

$$t = 29/6.5 = 4.5$$

On the 21st day:

$$SE_d = \sqrt{\frac{69 \times 31}{91} + \frac{93 \times 7}{91}} = 5.5, \qquad 93\% - 69\% \text{ observed difference}$$

$$t = 24/5.5 = 4.3$$

On the 28th day:

$$SE_d = \sqrt{\frac{74 \times 26}{98} + \frac{92 \times 8}{98}} = 5.2, \qquad 92\% - 74\% = 18\% \text{ observed difference}$$

$$t = 18/5.2 = 3.4$$

On the 35th day:

$$SE_d = \sqrt{\frac{76 \times 24}{111} + \frac{90 \times 10}{111}} = 4.9, \qquad 90\% - 76\% = 14\% \text{ observed difference}$$

$$t = 2.85$$

From the 111 positive cultures 26 or 23.6% were positive only by the pepsin-pentane procedure, and 11 or 9.9% by the 10% Na_3PO_4 procedure. Grossly this appears significant and the standard error of the difference of the two propertions shows the difference to be significant.

$$SE_d = \sqrt{\frac{23.6 \times 76.4}{111} + \frac{9.9 \times 90.1}{111}} = 5.1, \quad 24\% - 10\% \text{ observed difference}$$

$$t = 14/5.1 = 2.8$$

A quick diagnostic procedure in tuburculosis is the acid fast stain. The efficiency of the pepsin-pentane procedure is again indicated in the greater number of positive acid fast stains. Table 18 is a summary of the tabulated data which relates cultures and acid fast stain.

Table 18

ACID FAST STAINS AND CULTURES

	Acid Fast Stain				
	P+,T+	P+,T-	P-,T+	P-,T-	
Cultures					Totals
P+, T+	49	11	5	9	74
P+, T-	11	4	2	9	26
P-, T+	3	4	1	3	11
P-, T-	11	17	1	73	102
Totals	74	36	9	93	212

Some examples of the interpretation of Table 18 are: 49 is the number of specimens that were positive on acid fast

stains from the inocula of the concentration by both methods and were also positive on culture by both methods. The 5 at the top of the third column indicates that there were 5 specimens positive by stain with the 10% Na_3PO_4, negative by the pepsin-pentane method but positive by both culture procedures.

The 73 that were negative on both stain and culture may be due in great part to the many patients who had been under extensive treatment and were no longer expectorating tubercle bacilli. A second reason may be that one source of specimens was a chest clinic. The patients were admitted for any chest condition and a sputum routinely collected. It is quite likely also that a tubercular patient does not always expectorate tubercle bacilli.

Of the 212 specimens processed 110, or 52%, were positive on AFS by the pepsin-pentane procedure. By the 10% Na_3PO_4 procedure 83 of the 212, or 39%, were positive. We were interested in the significance of the difference of these percents.

$$SE_d = \sqrt{\frac{52 \times 48}{212} + \frac{39 \times 61}{212}} = 4.8, \quad 52\% - 39\% = 13\%$$

$$t = 13/4.8 = 2.7$$

From the 212 specimens processed 36 or 17% were positive only by the pepsin-pentane procedure, 9 or 4% were positive only by the 10% Na_3PO_4 method. The difference of these percents is highly significant.

$$SE_d = \sqrt{\frac{17 \times 83}{212} + \frac{4 \times 96}{212}} = 2.9, \quad 17\% - 4\% = 13\%$$

$$t = 13/2.9 = 4.5$$

From table 18 we have summarized further in table 19 the answers to questions such as: How many specimens were negative on stain by the pepsin-pentane procedure but positive on culture by the same procedure? Table 19 tells us 25. The number 25 was computed from four figures in table 18 (third and fourth columns and first and second rows).

Table 19

ACID FAST STAINS AND CULTURES, part 2

	Acid Fast Stain			
	P+	T+	P-	T-
Culture				
P+	75	67	25	33
T+	67	58	18	27
P-	35	16	78	97
T-	42	25	85	103

Comparative Study with Another Laboratory

A local hospital laboratory divided 48 sputa and treated half of each by their routine procedure (4% NaOH and centrifugation). Half of each specimen was sent to us for the pepsin-pentane procedure. Each laboratory prepared its own Lowenstein-Jensen medium. In table 20, negative cultures are indicated as such, but positive cultures are indicated by the number of days for the first appearance of growth.

Table 20

DATA OF COMPARATIVE STUDY WITH ANOTHER LABORATORY

	AFS		Culture			AFS		Culture	
	LU*	X*	LU	X		LU	X	LU	X
Spec.					Spec.				
1	-	-	-	-	11	-	-	-	-
2	-	-	-	-	12	+	-	-	-
3	+	+	22	22	13	-	-	-	-
4	+	-	-	-	14	+	-	-	-
5	-	-	-	-	15	-	-	-	-
6	-	-	-	-	16	+	+	-	-
7	-	-	-	-	17	-	-	-	-
8	+	+	16	22	18	+	-	-	-
9	+	-	18	-	19	+	-	-	-
10	-	-	-	-	20	+	-	-	-

(continued on page 80)

Table 20 continued

Spec.	AFS LU*	AFS X*	Culture LU	Culture X	Spec.	AFS LU	AFS X	Culture LU	Culture X
21	+	+	15	15	35	-	-	-	-
22	+	+	12	-	36	+	+	-	-
23	-	+	-	-	37	-	-	-	-
24	+	+	10	22	38	-	-	-	-
25	+	+	12	-	39	-	+	-	-
26	+	+	-	-	40	-	-	-	36
27	+	+	29	36	41	-	-	9	-
28	-	-	12	-	42	-	-	-	-
29	+	+	14	22	43	-	-	-	-
30	+	+	8	29	44	-	-	-	-
31	+	+	-	23	45	+	+	13	-
32	+	+	-	-	46	+	+	34	22
33	-	-	-	-	47	-	-	-	-
34	-	-	-	-	48	-	-	-	-

*LU = Loyola U. Dept. of Microbiology

*X = local hospital laboratory

Discussion of Comparative Study with Another Laboratory

In specimen 31 we failed to get a positive culture, although both laboratories had positive stains. Our records show that the specimen was received 5 days after collection. Weak viability and the prolonged delay may account for the loss. We have also observed that sputum specimens have an acid pH after such delays. We cannot account for specimen 40 and must simply attribute it to chance.

Table 21

SUMMARY OF COMPARATIVE STUDY WITH ANOTHER LABORATORY

	Positive Cultures							
	Only LU*	Only X*	LU & X	Total	Total LU	Total X	LU%	X%
Days								
14	8	0	0	8	8	0	100%	0
21	10	0	2	12	10	2	83%	17%
28	7	2	5	14	12	7	86%	50%
35	6	3	7	16	13	10	81%	63%

*LU = Our Laboratory, pepsin-pentane

*X = Another Laboratory, 4% NaOH and Centrifugation

The interpretation of table 21 is the same as for table 17. The 14 and 21 day period are grossly significant. An analysis of the 28 and 35 day periods are presented.

On the 28th day:

$$SE_d = \sqrt{\frac{86 \times 14}{14} + \frac{50 \times 50}{14}} = 16.3, \quad 86\% - 50\% = 36\% \text{ observed difference}$$

$$t = 36/16 = 2.24$$

On the 35th day:

$$SE_d = \sqrt{\frac{81 \times 19}{16} + \frac{63 \times 37}{16}} = 15.6, \quad 81\% - 63\% = 18\% \text{ observed difference}$$

$$t = 1.2$$

From the "t" test we see that there is no significant difference between the 81% and the 63%. We consider that the number of specimens in this period was too small. In the comparative study within our own laboratory significant results only became apparent after a representative number of tests had been performed. We compiled similar summaries in order to show the trend in even a small number of specimens.

Table 22

ACID FAST STAINS AND CULTURES

	Acid Fast Stain				
Culture	LU+, X+	LU+, X-	LU-, X+	LU-, X-	Totals
LU+, X+	8	-	-	-	8
LU+, X-	3	1	-	2	6
LU-, X+	1	-	-	1	2
LU-, X-	3	7	2	20	32
Totals	15	8	2	23	48

The interpretation of table 22 is the same as table 18.

Chapter VI

GENERAL DISCUSSION

Recovery of viable tubercle bacilli for a positive diagnosis of tuberculosis has many obstacles. If one considers that the tubercle bacilli have been removed from their parasitic environment long before the specimen is obtained, delay in processing, deleterious treatment, and finally artificial culture medium, it is simple to understand that some tubercle bacilli may fail to grow. We have been concerned with the problem of processing preliminary to culture. Dubos (1954) considered present day procedures for cultivating tubercle bacilli from clinical specimens as "barbarous" treatment that kill many of the bacilli. Probably no procedure will be 100% effective in the recovery of the tubercle bacilli. We believe that the procedure that we have developed is an improvement over other procedures in common use.

We have presented significant experimental evidence that the pepsin-pentane procedure is best for the processing of sputum. We have also used this procedure successfully on bronchoscopy specimens. These specimens are quite mucoid. Digestion need not proceed for more than an hour. We have found that fresh specimens, although mucoid, digest quickly. They are also comparatively free of contamination.

The procedure lends itself very well for digestion of mucus in gastric juice with one modification. We use the 0.5% pepsin without citric acid. This pepsin solution is usually at pH 4.2. In the small number that we have processed the gastric juice had sufficient acidity to activate the pepsin.

The pepsin-pentane procedure may also be used very satisfactorily to recover tubercle bacilli from lung tissue. The specimens may be best collected in sterile saline at the postmortem table and the surgical gross examination table. In the bacteriology laboratory the sections are further cut into small fragments with scalpel and scissors to speed the enzyme digestion. The specimen is placed into the incubator and the procedure is the same as outlined.

We propose the use of laryngeal swabs with the pepsin-pentane procedure also. The reports of Armstrong (1951), Saenz (1952 & 1953), Chaves (1953) are comparative studies to show the efficiency of the laryngeal swabs over gastric washings. In all of these either 10% Na_3PO_4 or 4% NaOH was the digestant. Mankiewicy (1953) used gelfoam swabs which readily dissolved in 3% NaOH. We have found that gelfoam (UpJohn Co.) dissolves within 20 minutes at room temperature with the pepsin digestant.

For spinal fluid, cystoscopic and catheterized

urine, joint and thoracentesis fluids, etc., we omit the pepsin digestion. Pentane concentration is sufficient. (Steps 3 and 4 of the procedure on page 58) For these specimens we are also interested in membrane filtration procedures as described by Trompke and Kroger (1952), Muller (1952), and Morgante and Murray (1955).

From table 18 we determined that 52% of the 212 specimens were positive by acid fast stain with the pepsin-pentane procedure whereas 39% were positive by stain with Na_3PO_4 procedure. Throughout our work the two smear preparations were made on the same slide so that staining was always performed simultaneously. Frequently we noticed a contrast in the intensity of the stain reaction. The pepsin-pentane procedure consistently gave an intense reaction, but the 10% Na_3PO_4 was not dependable. The latter frequently appeared dull or faded. We believe this factor is significant for our procedure. Spendlove, Cummins, and Patnode (1949 a) also observed loss of acid-fastness with NaOH and Na_3PO_4 treatments. This could be an explanation for superior results from acid fast stains by the pepsin-pentane procedure.

Early diagnosis is a factor in efficiency of a procedure. With the pepsin-pentane procedure we have not

only obtained more positive cultures but significantly earlier results. From table 17 we see that in 14 days 64, out of a possible 100, were positive by the pepsin-pentane procedure whereas 44 out of 85, or 52%, were positive by the Na_3PO_4 procedure in the same time. In 21 days 85% of the pepsin-pentane positives had already appeared and it took 28 days for 73 out of 85, or 86%, of the Na_3PO_4 positives to appear.

We expect culture medium to speed up even these early observations. For this purpose we have done some work with tetrazolium salts as part of culture medium and have observed growth of tubercle bacilli by their reduction of the salts to a red formazon within three days. Much work remains to be done but it looks very promising. Vandiviere, Gentry, and Willis (1952), and again Willis (1953), and Koch-Weser and Ebert (1955) studied tetrazolium salts in relation to tubercle bacilli.

Summary and Conclusions:

We have presented a procedure for the recovery of tubercle bacilli from clinical specimens which includes a pepsin-citric acid digestant and concentration of the tubercle bacilli at an interface by thorough agitation with pentane. Analysis of data in a comparative study of the new procedure and 10% Na_3PO_4 procedure with 212 specimens shows that the pepsin-pentane procedure gives:

a) a higher percentage of positives

b) more positives from direct acid fast stains of the concentrate used for inoculation

c) earlier observation of culture growth.

LIST OF TABLES

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APPROVAL SHEET

The dissertation submitted by Sr. M. Imogene Palen has been read and approved by seven members of the faculty of the Graduate School and the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

May 22, 1956
Date

Einar Leifson
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