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Studies on the Dubos-Middlebrook Cytochemical Virulence Test for Mycobacterium Tuberculosis

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STUDIES ON THE DUBOS-MIDDLEBROOK CYTOCHEMICAL VIRULENCE TEST FOR MYCOBACTERIUM TUBERCULOSIS

by

Harry Youmun Wong

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A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Harry Youmun Wong was born in Ewa, Oahu, territory of Hawaii, September 21, 1929.

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INTRODUCTION

The laboratory diagnosis of tuberculosis deals with the identification of virulent tubercle bacilli. Toward this end three procedures are available to the bacteriologist: 1) Acid fast staining of a smear of the clinical material. 2) Culturing the clinical material on appropriate media to isolate the causative agent, and 3) Inoculation of guinea pigs with the clinical material. In cases where the clinical evidence of tuberculosis is clear-cut the bacteriologist need perform only the first procedure which is the simplest and quickest of the three procedures. However, in most cases more observation of acid fast organisms under the microscope is not enough and the organisms must be isolated either by culturing on artificial media or by inoculating guinea pigs. The inoculation of guinea pigs has the advantage over the artificial media procedure in that it also constitutes a virulence test.

It would not be essential to perform the guinea pig virulence test if all acid fast organisms cause tuberculosis in man. There are many types of saprophytic acid fast organisms and non virulent acid fast organisms which are found most anywhere. Due to improvements of culture media and methods for isolation of acid fast organisms and the high cost of using guinea pigs there has
been a tendency to omit the use of guinea pigs and concentrate on artificial culturing of the tubercle bacilli. However, it is essential that we have a laboratory means of distinguishing virulent acid fast organisms from non virulent acid fast organisms.

In the last decade or so several in vitro virulence tests have been proposed which are more rapid and cheaper than the guinea pig test. Of these the dye test of Dubos and Middlebrook seems to be the most promising and deserving of our attention.
STATEMENT OF THE PROBLEM

The problem of this investigation is to obtain a better understanding of the mechanism behind the Dubos-Middlebrook cytochemical virulence test for Mycobacterium tuberculosis and possibly to obtain a simplified modification of this cytochemical virulence test.
REVIEW OF THE LITERATURE

Before reviewing the literature on the neutral red test several factors in relation to the problem of virulence determination in tuberculosis will be discussed. With this in mind the author will discuss the organisms that may be confused with the tubercle bacilli and the various tests proposed to distinguish between the virulent tubercle bacilli and the saprophytic and non virulent atypical forms.

The term "tubercle bacillus" is restricted to four types of pathogenic acid fast bacilli, the human, bovine, avian and murine. Human tuberculosis is usually caused by the human and bovine types. The other members of the family Mycobacteriaceae all possess the staining characteristics and general morphology of the tubercle bacillus. In general they grow luxuriantly and comparatively rapidly on common laboratory media even at room temperature and below. The characteristics which are considered by most, and specifically cited by Hastings and McCarter, as prerequisites before an organism can be considered a human or bovine tubercle bacillus are: 1) optimum growth at 37 C; 2) slow growth, that is, taking approximately one week to show visible growth; 3) no growth on plain agar media; 4) grows only upon the surface of media;
5) will sensitize an animal to subsequent inoculation with a virulent tubercle bacillus; 6) pathogenic for guinea pigs if human strains and for both guinea pigs and rabbits if bovine strains.

It has been known for a long time that the saprophytic acid fast organisms are ubiquitous in nature. Reports of the isolation of atypical mycobacteria from human sources are appearing in the literature with increasing frequency. The questions arises as to the difference between the saprophytic acid fast organisms and the atypical mycobacteria. Both types being so far considered non pathogenic for guinea pigs and human beings. Both types are usually pigmented and usually grow faster than the tubercle bacillus. The difference is not clear-cut. The saprophytic forms are common in nature, associated usually with cold blooded animals or free living in water or soil. They may also be found on man. They produce visible growth on laboratory media within 24 hours. The atypical types of mycobacteria are unusual either in shape, size, staining quality or colony characteristic, and which are reported as being found primarily in association with patients in the hospital who show symptoms of tuberculosis. They grow more rapidly in laboratory than the virulent tubercle bacilli but usually at a rate intermediate between the virulent tubercle bacilli and the saprophytic types. They are non pathogenic for guinea pigs and rabbits but are pathogenic for mice and hamsters when given in large doses.

A non pathogenic acid fast organism was first discovered in a fish by Bataillon, Dubard, and Terne in 1897. This bacillus
grew rapidly at 100C and was not pathogenic for guinea pigs, rabbits or fowls. In 1889 Sibley described acid fast organisms in snakes, and Hansemann in 1903 reported them in a python, but cultures were not obtained by these workers.

One of the better known saprophytic organisms isolated from cold blooded animals is the turtle bacillus discovered by Friedmann in 1903. This organism was utilized unsuccessfully by Friedmann as an antituberculosis vaccine. This organism grows equally well at 22 as at 370C and is not pathogenic for guinea pigs.

Another group of saprophytes are the various acid fast organisms isolated from grasses, seeds and milk. Examples of these are the timothy bacillus of Moeller (1899), bacillus tuberculoides of Beck (1905), Korn (1899) and Tobler (1901). All grow well at room temperature. Some produce an orange or reddish pigment. Otherwise they appear much the same as cultures of tubercle bacilli. They are rapid growers.

Included in the saprophytic acid fast group is the smegma bacillus which may be mistaken for the true tubercle bacillus especially in examinations of urine and feces. Alvarez and Tavel discovered the smegma bacillus in 1885 and seem to be a normal inhabitant of the orifices of animals and man where sebaceous grease is abundant, hence the name smegma bacillus. Morphologically and culturally it is similar to the tubercle bacillus. Gordon and Smith (1933) in an attempt to describe various species of saprophytes describes the smegma bacillus as usually slender, of
varying length, curved and beaded. Growth on glycerol agar in 2 to 3 days is spreading, finely wrinkled, waxy, creamy yellow to pale orange.

In 1909 Brem made smears from tap water and distilled water in his laboratory and found numerous acid fast organisms. Upon inoculation of guinea pigs they did not cause tuberculosis in the animals. Care must be taken in diagnosing tuberculosis merely by smear and acid fast staining since the organisms may be saprophytes from the water used in staining.

In 1931 Frey and Hagan studied soil samples from various parts of the world and cultured from every one of one hundred samples acid fast organisms.

According to Willis and Cummings (1952) the saprophytes may be found in man particularly in nasal discharges, sputums, ear waxes and stool.

In 1939 Lester isolated 130 strains of saprophytic acid fast bacilli from 26,343 human specimens. This being an incidence of 0.5%. Of the 130 specimens 75 or 57% were from stomach washings, 13 or 10% from sputum and 24 or 18.4% from urine.

In a total of 5000 sputum cultures Pinner (1935) found that 0.9% showed growth of saprophytic acid fast bacilli.

Willis and Cummings (1952) quoted Smith as having found slightly more than 1% of the cultures obtained from sputum were acid fast saprophytes.

Many saprophytes have been isolated throughout the years
but it is the opinion of Willis and Cummings (1952) that they may all have a common origin. It is believed by these authors that the turtle, frog, snake and fish acquire their acid fast saprophytes from the grasses in the water in which they live and that horses and cattle acquire their saprophytes by ingestion of phlei bacilli along with the hay that they eat.

The literature on the occurrence of atypical mycobacteria found in human beings whether normal or diseased is voluminous and no attempt is made here to review it. The works of Pinner (1935), Tarshis (1952) and Frisch, Wolinsky (1957) et al, Schmidt (1957) et al, Timpe and Runyon (1954), Crow (1957) et al, Wison and Morton (1956) should be consulted. Most of these studies deal with organisms which are somewhat different culturally from the tubercle bacilli. However no agreement has been reached by bacteriologist on the classification of acid fast bacilli labeled as atypical on culture. As revealed by Crow (1957) et al reports from widely scattered sections of the country and even from local areas show that there are differences in cultural growth and other characteristics.

Atypical mycobacteria have been isolated from patients with lesions considered tuberculous, while others have reported such organisms isolated from patients with pulmonary disease considered nontuberculous. Isolation of atypical forms have also been found in persons with normal chest picture. There appears to be no constant human source for these organisms and although isolated
primarily from sputum, they have been obtained from skin, tonsils, ears, nasal secretions, mucous membranes, urine and exudates from various lesions.

Yegian and Budd (1953) and Griffith (1916) described cases where acid fast organisms that were culturally and morphologically identical with human tubercle bacilli were isolated from human beings. They were distinguished solely on their inability to cause tuberculosis in guinea pigs.

The last group of organisms which should be considered are the initially virulent tubercle bacilli which upon maintenance in laboratory media for a number of years developed into avirulent strains. They no longer have the ability to cause progressive tuberculosis in the guinea pig. The H37Ra, RIRa, JHI6Ra avirulent strains are examples of these initially virulent human strains. The history of these strains is described by Steenken and Gardner (1946). They represent an important group because they are probably just a few examples of avirulent variants that may also be found in nature.

While the guinea pig virulence test is the standard and most reliable test in use today the disadvantage of such an in vivo animal virulence test is the necessity of maintaining animals and the time required before results of the test can be obtained. Within the last decade or so various in vitro tests have been devised to get away from these two objections of the guinea pig virulence test.
In 1940 Schain and Petroff found that growth of tubercle bacilli was partially inhibited in solid media containing 15 to 25 units of diphtheria antitoxin per ml. In 30 to 60 units per ml the multiplication of the bacilli was inhibited entirely. It was found subsequently that there is an inverse ratio between the virulence of acid fast organisms and amount of antitoxin required to inhibit its growth. Virulent tubercle bacilli such as H37Rv were inhibited in concentrations of 30 to 60 units whereas avirulent tubercle bacilli such as H37Ra were inhibited in concentrations of 75 to 90 units and acid fast saprophytes such as M. smegmatis and M. phlei were not inhibited in concentrations of up to 125 units of antitoxin per ml of medium. This relationship was suggested as being useful in determining virulence of acid fast organisms.

However in 1946 Wagley and Steenken Jr. attempted to repeat the experiments of Schain and Petroff but found little correlation between virulence and inhibiting concentration of diphtheria antitoxin. The effective inhibitory concentration of antitoxin varied from one strain to another and over a wide range, irrespective of virulence of the cultures. There were no marked zones of inhibition produced by the antitoxin that would be helpful in differentiating between virulent and avirulent human tubercle bacilli.

Middlebrook, Dubos and Pierce in 1947 working with the standard H37Ra and H37Rv strains and other virulent and avirulent
stock strains discovered that when the virulent strains were inoculated in Dubos tween Albumin media with 0.02% of tween 80 it gave rise to growth consisting of bundles, ropes or cords of bacilli in which the long axis of each cell was oriented parallel to the long axis of the cord. This tendency of the virulent variants to form cords can be completely inhibited by cultivation in media containing 0.05% or more of tween 80; under these conditions the culture becomes highly diffuse and consist predominantly of isolated bacterial cells. Transfer of this highly dispersed culture to a medium containing no tween 80 or a low concentration of this substance results again in the formation of cords. This cord formation was not considered an artifact of cultivation since it was found that short but definite cords and bundles of tubercle bacilli arranged themselves in parallel in the brain tissue of mice infected intracerebrally with high dilutions of a diffuse culture of H37Rv. It was also demonstrated that cords would form in the yolk sac of chick embryos infected with H37Rv or with other virulent cultures of mammalian tubercle bacilli. In contrast the authors found that the avirulent variant, H37Ra has never been observed to form definite cords under any condition of growth. It grows as non-oriented clumps. However it is the personal observation of the present author that the avirulent H37Ra will form cords when inoculated into normal enembryonated eggs. Middlebrook et al had also observed that the strain RIRv and the BCG strain which possess low but definite virulence would form cords in media
with no tween 80 but not in media containing more than 0.01% of this substance. Thus it takes a lesser amount of tween 80 to inhibit cording in the less virulent strains. This is an implication that the less virulent the strain the less strong or tight is the cording. However it should be pointed out that Bretey et al in 1945 observed cording in rapidly growing cultures of saprophytes. On the other hand Middlebrook et al ignored this observation on the basis that the organisms of Bretey et al was so different in characteristics from the H37Rv and H37Ra that they could never be confused with these standard virulent strains.

Although Middlebrook et al is credited for relating cording with virulence of the acid fast organisms other workers have also observed that cultures of mammalian tubercle bacilli may grow in the form of microscopic serpentine cords.

In 1948 Bloch noticed that the distilled water extracts of chick embryos when incorporated into the media would cause avirulent H37Ra to form well defined cords, and growth assumes again the usual unoriented pattern as soon as the strain is returned to media without the chick embryo extract.

In 1952 Yegian and Kuring found that the addition of a small amount of normal human blood serum or plasma to liquid tween albumin gives rise to marked serpentine growth of virulent tubercle bacilli. Normal sera from different animals proved to be as effective as human serum. One of the strains studied by these authors failed to show agreement between growth pattern and virulence.
This strain a streptomycin-dependent variant derived from the parent virulent H37Rv strain, proved to be a good cord former although it was not virulent for guinea pigs even when the animals were treated with streptomycin. The authors concluded that in general while all cord formers are not necessarily virulent, all virulent strains are cord formers.

This enhancement of cord formation by normal sera could be explained somewhat by the observation of Davis and Dubos in 1946 that unheated sera or plasma has esterase activity for tween 80, splitting the oleic acid from the rest of the molecule and thereby destroying the surface active effect of tween 80. This surface active effect of tween 80 tends to disperse the tubercle bacilli and prevents clumps to be formed in the liquid media.

In 1950 Bloch noticed that when wet bacilli are suspended in hydrocarbons such as paraffin oil or petroleum ether, cording is disrupted. Thus he used petroleum ether to extract the cord factor. He put the virulent bacilli into petroleum ether with stirring for five minutes, spun down, filtered and took the supernatant and evaporated off the hydrocarbon. The sediment was dissolved in ethyl ether and then precipitated with ethanol. This extract was colorless, amorphous and waxy, melting at 30-31 C. It was soluble in ether, petroleum ether, paraffin oil, normal hexane heptane, chloroform, benzene, and dioxane. It was insoluble in ethanol, methanol, acetone, water and pyridine. It was acid fast and inhibited the migration of leucocytes in vitro which is a
characteristic of virulent tubercle bacilli.

In 1953 Asselineau, Lederer and Bloch using the method of Anderson in extracting lipids from mycobacteria found that the so called cord factor could be extracted out by this method and the activity was found in the wax C fraction. This wax C fraction is in general obtained by first extracting the tubercle bacilli with ethanol-ether. The remaining cells are then taken and extracted with chloroform. The chloroform extract is then treated with hot acetone and this substance which is soluble in hot acetone is called the wax C fraction.

In 1933 Aksianzew, using the standard Thunberg technique, reported that virulent mycobacteria decolorized methylene blue more rapidly than did BCG. In 1946 Roulet, Sydler, and Zeller, studying the enzymatic degradation of certain oxyacids by mycobacteria, observed incidentally that M. smegmatis decolorized methylene blue at a more rapid rate than did the human tubercle bacillus.

In 1950 Bloch while studying the behavior of different dehydrogenases from tubercle bacilli using the Thunberg methylene blue method, observed that methylene blue was more quickly decolorized by suspensions of avirulent than of virulent tubercle bacilli. In the presence of different substrates such as glycerol, methylene blue was decolorized only by washed suspensions of saprophytes, avitulent tubercle bacilli, and strains of BCG. In some instances, the age of the culture was important for the outcome
of the experiment. Whereas cultures of avirulent or saprophytic strains decolorized methylene blue at the same rate regardless of the age of the culture, suspensions of BCG and of some strains of virulent bacilli did not decolorized methylene blue as long as they were obtained from young cultures, but began to do so when obtained from older cultures. The methylene blue decoloration has the character of an enzymatic reaction. The temperature optimum of the reaction is 39°C with pH optimum between pH 7 and 8. The factor is inactivated by heating the bacilli at 56°C for 3 minutes. Although suspensions of virulent bacilli grown in media containing a high concentration of tween 80 will decolorized methylene blue. The authors state that while saprophytic mycobacteria decolorize methylene within 3 minutes, and strains of avirulent bacilli or BCG within two to 15 minutes, the young virulent strains do not decolorize methylene blue within sixty minutes. Here the factor of time is considered important.

In 1951 Wilson, Kalish and Fish made potentiometric studies on various strains of mycobacteria. Using the Beckman Model G pH meter adapted to determine oxidation reduction potentials it was found that all virulent strains of mycobacteria such as the H37Rv and bovine strain Ravenal gave almost identical oxidation-reduction curves throughout the growth period. The H37Ra and BCG strains presented curves different from those produced by the virulent bacilli or acid fast saprophytes. From the same studies of approximately -0.2 volts would be decolorized by
avirulent strains but would not be affected by virulent strains of mycobacteria. Four redox dyes were selected for use in a dye decolorization test; all of the dyes having an oxidation-reduction potential of -0.2 volts or thereabouts. One and a half tenths of a ml of the four dyes were placed in three rows of depressions of a white porcelain spot plate. To the first row nothing was added. To each of the four depressions of the next row was added approximately 5 mg of the avirulent saprophyte Mycobacterium phlei scraped from a slant of Lowenstein medium. The same amount of the virulent H37Rv was added to the last row. The suspensions were mixed thoroughly by means of a wooden applicator. Within 15 minutes, the four dyes containing the saprophytes were completely decolorized. The dyes containing the avirulent organisms retained some color. With this method 34 saprophytes isolated from pathologic material and which showed typical cultural characteristics gave non-virulent reactions. They decolorized the dyes. However, 7 out of 230 wild strains of human tubercle bacilli gave reactions typical of virulent strains. They did not decolorized the dyes. A drawback of this test aside from the inconsistent results just mentioned, is that mixed cultures containing both virulent and avirulent organisms may give a reaction indicative of only avirulent cells and therefore mislead the investigator. Another drawback of the test is that the amount of cells used is critical. If less than 5 mg of saprophytes are used a false positive reaction may occur; more than 10 mg of virulent cells on the other hand may
cause decolorization of the dyes. Cultures used must be viable with potential danger to the person performing the test. It was admitted by the authors that the test in its present form is unsatisfactory for routine use as an *in vitro* virulence test to replace animal inoculations because of the false reactions.

In 1953 Whitehead et al studied 154 strains of mycobacteria in regards to their ability to hydrolyze phenolphthalein disulphate. All 51 strains of the saprophytic studied showed ability to hydrolyze phenolphthalein disulphate. The hydrolysis releases phenolphthalein into the media which is detected by addition of NaOH dropwise. Under the same conditions all the strains of *Mycobacterium tuberculosis* (human type) and most of the bovine types failed to hydrolyze the compound to a detectable extent. However, 16 of the 51 strains of saprophytes showed only very weak activity and the standard H37Ra strain showed no activity. The authors concluded that more work need to be done before this test can be applied as an aid in the determination of type or virulence of mycobacteria.

In 1952 Singer and Cysner devised a simple test for urease activity. The growth from solid media is suspended in a 0.5% urea solution and ammonia production determined at various times with Nessler's reagent. Eleven strains of tubercle bacilli, and 53 strains of saprophytes were studied for urease activity. It was found that although all of these strains had urease activity it was possible to differentiate the tubercle bacilli from the
saprophytes by the rate of breakdown of urea. Those strains which show ammonia production in six hours or less should not be considered tubercle bacilli; none of the 11 strains of tubercle bacilli showed ammonia in less than 6 hours. However, the slow splitting of urea is no proof that the strain concerned is a tubercle bacilli since H37Ra showed ammonia only after 2 days. Twenty one saprophytes showed ammonia only after 24 hours.

Of all the in vitro virulence tests devised in the past decade the neutral red test seems the most promising and the one which is accepted most widely.

In 1948 Dubos and Middlebrook using the standard H37Rv and H37Ra strains found that when virulent tubercle bacilli were placed in an alkaline barbiturate buffer of pH 9.4 to which is added a small amount of neutral red the cells will bind the dye and become red. This occurs usually within half an hour to an hour. At that time the dye remaining in solution is yellow whereas that fixed on or in the cells is red. In the case of the avirulent H37Ra, no significant fixation of the dye occurs and the cells are colored amber like the supernatant solution. It was stated that many unrelated substances in the media can mask the reaction and therefore the cells should be washed twice with 50% methanol at 37°C to eliminate these substances from the cells.

In 1949 Dubos and Suter discovered that cultures grown in the presence of high concentrations of asparagine (0.5%) failed to bind neutral red unless they were prewashed thoroughly with 50%
methanol. On the contrary, cultures grown in media in which asparagine has been replaced by glutamic acid, give a positive neutral red test even without prior washing. It was also discovered that it is not asparagine per se which exerts this inhibitory effect but some metabolic product of the bacilli growing in the presence of the asparagine. It was shown that ammonium salts inhibit the virulent cells from binding the dye unless they were washed thoroughly with methanol. These findings apparently explained the reason that washing of the cells with 50% methanol is necessary for a proper result.

In the same year Haudurou and Pasternak in France studied 67 strains of virulent tubercle bacilli and 67 strains of saprophytic acid fast organisms and found all virulent strains to give a positive neutral red reaction whereas the saprophytic strains gave a negative reaction. Besides, this, 4 pathogenic and 4 non-pathogenic actinomycoses, 4 virulent toxigenic diphtheria and 4 pseudodiphtheria, 6 coagulase positive staphylococci, 4 coagulase negative staphylococci were studied and found to give negative neutral red reactions. It was interesting that 4 acid fast strains which showed cultural characteristics like the saprophytic strains but giving a positive guinea pig virulence test also gave a positive neutral red test, which points out the close correlation of guinea pigs test with the neutral red test regardless of the cultural characteristics of the organisms.

In 1950 Richmond and Cummings in evaluating the neutral
red test studied 19 strains of acid fast bacilli. There were 13 virulent strains, one attenuated, and 5 saprophytic strains. It was found that the degree of intensity of redness was not correlated with degree of virulence in the guinea pig. The redness was constant for each strain but varied from strain to strain. All strains virulent for the guinea pig was neutral red positive but one saprophytic strain from a radish gave an intense neutral red test. There was good correlation between cording and a positive neutral red test. The strains recently isolated from human infection showed more intense color reactions than did the stock virulent strains which had been carried for sometime in the laboratory. It was interesting to note that the standard H37Ra strain which is considered avirulent gave a slightly positive neutral red test.

Along with studying the neutral red test Richmond and Cummings also studied cording and found that there was very little correlation between cording and animal pathogenicity. However, it was stated that this was probably so because the Dubos tween albumin media in which the cells were cultured consisted of 0.02% tween 80 and Dubos had specifically stated that if the media consisted of more than 0.01% tween 80 cord formation is poor.

Morse, Dail and Olitsky in 1953 found perfect correlation between neutral red test and guinea pig virulence in 168 acid fast strains culturally consistent with *Mycobacterium tuberculosis*. Twelve acid fast chromogens gave negative red test as did the H37Ra strain used as control. Four human, 3 avian and 2 bovine
lyophilized stock mycobacteria gave positive neutral test whereas lyophilized stock *M. laticola*, *M. stercosi*, *M. leprae* and *M. phlei* gave neutral red negative reactions. There were two acid fast strains which gave neutral red positive tests but failed to infect the guinea pig in repeated tests. However, when these two strains were inoculated into chickens the results indicated that they were apparently avian strains which ordinarily are non pathogenic for guinea pigs. It is pointed out that if the neutral red test had not been available, these two strains, on the basis of their cultural characteristics and guinea pig tests would have been classified as non pathogenic mycobacteria. However, this two strains were guinea pig positive when first isolated. The authors conclude that the neutral red virulence test is just as indicative of virulence as the conventional inoculation of experimental animals. The neutral red test however has the advantage of saving appreciable time in the diagnosis of tuberculosis, and of reducing the health hazard to laboratory personnel which is present when animals are used for pathogenicity studies.

In 1954 Hughes, Moss, Hood and Henson reported a study of 191 cultures of acid fast organisms isolated from patients. Of these 189 were neutral red positive and virulent for guinea pigs. The remaining two were found to be acid fast strains of *Norcardia* species. Known avirulent mycobacteria such as *Norcardia asteroides*, acid fast non pathogens, and various gram positive and gram negative cocci and bacilli, yeast and molds all gave negative neutral
Robbins et al in 1955 studied a series of virulent tubercle bacilli, saprophytes and chromogens and found only the virulent tubercle bacilli to give positive reactions with the neutral red test.

With the increase use of antibiotic therapy in tuberculosis, especially with isoniazid, there is appearing more and more in the literature reports of the relationship of isoniazid resistance and guinea pig avirulence. In this respect Patnode, Dail, and Hudgins studied the relationship between isoniazid resistance, guinea pig virulence and the neutral red test. It was found that all strains of tubercle bacilli isolated from patients receiving isoniazid, and resistant to isoniazid to varying degrees, were attenuated in virulence for the guinea pig. However these resistant organisms did not differ from non resistant typical virulent strains in giving neutral red positive reactions or to form cords in Dubos broth without tween 80. These isoniazid resistant organisms also showed a negative oxidation-reduction reaction which is typical of virulent mycobacteria.

In 1955 Wayne studied the neutral red test on organisms cultivated on millipore filter discs that were placed on top of Lowenstein-Jensen egg medium. The supposed advantage of this was to have a method whereby you could observe cording and neutral red test very early in the growth of the organisms since the millipore disc can be taken off and examined under the microscope to see if
growth was present and if the organisms were corded and neutral red positive. The neutral red had to be modified somewhat to make it applicable to the growth on the filter disc. This was done by staining the growth on the filter disc with a saturated solution of neutral red in 95% ethanol for 10 minutes, then decolorize with a saturated aqueous sodium carbonate solution for 5 minutes and finally removal of the excess reagents with 95% ethanol for 1 minute. The stained growth on the filter disc was then dried and mounted on a slide in permount fluid under a cover slip and observed under the microscope. It was stated that six days old growths or older could be studied by this method. However it should be noted that with this method strains of BCG, 1 strain of M. ulcerans, 1 strain of a saprophyte and 4 strains of attenuated human mycobacteria were neutral red positive. No virulent mycobacteria were neutral red negative.

Independently in the same year Krasnow applied the neutral red staining technique to cultures of mycobacteria grown on filter paper. The method is essentially the same as that of Wayne except that Whatman No. 1 filter paper was used instead of the millipore filter disc. The authors did a parallel study of more than 50 mycobacteria cultures using both the millipore filter and filter paper methods and found both to give identical results. Based upon the neutral red reaction and cording, all of the mycobacteria tested could be divided into four groups. Neutral red positive and corded, neutral red positive but not corded, neutral
red negative but corded, and neutral red negative and not corded. All the virulent stock strains were neutral red positive and corded. Four strains of BCG also fell into this group. Two avirulent variants of \textit{M. tuberculosis} var. \textit{hominis}, \textit{M. ulcerans}, the radish saprophyte and 10 of 11 strains of chromogens were found to be in the group of neutral red positive but non corded. \textit{Mycobacterium phlei} was the only organisms which exhibited cord formation and yet was neutral red negative. The authors stressed the use of parallel orientation rather than serpentine growth as a criteria of cording. It is believed by the authors that the microcolonial test used is useful as a means of interpreting the history of an organism. That is, a mycobacterium cultured from a patient, and which is neutral red positive and corded is probably derived from a virulent mammalian tubercle bacillus, even if such an organism is presently incapable of producing progressive disease in animals. In this fashion they explain away the fact that 4 strains of BCG that they studied were neutral red positive since BCG historically came from a virulent bovine culture. The writers concluded that a strain of mycobacterium negative by one or both criteria (neutral red reaction or cord formation) is not a virulent tubercle bacillus. It should be noted that the results obtained by Krasnow should not be classified under the neutral red test as originally proposed by Dubos and Middlebrook as the neutral red test as done by Krasnow and by Wayne is modified in that the concentrations of the neutral red, the buffer and the time each is applied to the
organisms differ. This in essence may make the neutral red test as done by Dubos and Middlebrook and the neutral red test as done by Krasnow and by Wayne two different tests entirely. It is conceivable that it is possible to change the concentrations of the neutral red and buffer in the original neutral red test to such an extent that all organisms including the saprophytic acid fast organisms would give a positive red color.

Buchanan in 1955 modified the neutral red test by eliminating the 50% methanol wash and reading the reaction after 1 hour incubation at 37°C. Of 121 strains of M. tuberculosis, 120 gave a positive reaction as did the vole bacillus and BCG. The avirulent and chromogenic cultures gave a negative reaction. The results on the whole were like those of most other workers. There was close relationship between the neutral red test as performed according to the original Dubos and Middlebrook method and by the modified method where the washing procedure was eliminated. Of 65 strains of mycobacteria compared in this way only 2 strains were negative by the modified method but positive by the standard method. The modified test was performed on growth taken from Lowenstein-Jensen cultures whereas the work first done by Dubos and Middlebrook liquid Dubos tween albumin media was used to culture the organisms. In a similar experiment done independently by the present writer it was found that elimination of the methanol wash may work if the test is read after several hours at room temperature instead of after 1 hour. Elimination of the methanol
wash will not work if the cultures are from liquid Dubos tween albumin media.

After Hauduroy introduced the neutral red test in Europe, Viallier and Tigaud in 1953 found that they could quantitate the neutral red reaction by using the spectrophotometer to measure color intensity. They also found that by using a certain proportion of neutral red-buffer they could observe the neutral red reaction under the phase contrast microscope. Using this method they found they could dispense with the 50% methanol wash. With the phase microscope it was also possible to determine the percentage of colored or stained cells in a cell suspension at various stages of growth. It was found that in the early lag phase there was a large proportion of unstained cells. The authors also pointed out that culturing the virulent cells in deficient media affected its ability to give the neutral red test.

In 1953 Desbordes introduced the Nile blue virulence test which is essentially the neutral red test except that a different basic dye, nile blue is used and at a pH of 11 instead of pH of 9.4. Here the virulent cells are colored blue while the avirulent cells are colored rose.

There are two theories which are proposed for the mechanism of the neutral red test. The first is that proposed by Desbordes and Fournier in 1950 which leaves us to understand that since neutral red is a vital stain and a redox indicator the neutral red test depends on a intracellular oxidation-reduction poten
tial differing with the strain used.

The second explanation is proposed by Asselineau and Lederer in 1952. Here the authors leave us to believe that a certain wax fraction purified after the method of Anderson, which is insoluble in hot acetone and called the wax D fraction, is responsible for the neutral red test. The authors have found that this fraction varies in concentration in various strains of mycobacteria, the virulent strains having a greater concentration than the avirulent strains. The Dubos and Middlebrook neutral red test was applied to the wax D fraction and the wax D fraction of the virulent strains gave an intensely red reaction.
CHAPTER III

THE IMPORTANCE OF THE 50% METHANOL WASH IN
THE DUBOS-MIDDLEBROOK TEST

Dubos-Middlebrook Test:
a) spin down cells from 2 week old culture in Dubos medium and
discard supernatant.
b) wash cells twice with 50% methanol, cells being maintained for
one hour at 37°C in the methanol each time before being sepa-
rated by centrifugation.
c) resuspend washed cells in 5 cc of 5% NaCl-1% sodium barbitu-
rate buffer.
d) add 1 cc of 0.01% aqueous neutral red solution and observe at
room temperature.

Using this procedure the H37Rv cells should be red and the H37Ra
cells should be white within the hour.

Two week old cultures of H37Rv* and H37Ra* Dubos medium
were spun down, supernatants discarded and 5 cc of barbiturate
buffer was pipetted into the H37Rv and H37Ra tubes followed by one
cc of 0.01% aqueous neutral red. After one hour at room tempera-
ture both the H37Rv and H37Ra cells were positive, that is, were
colored red to pink.

The experiment was repeated using surface growth from
Loewenstein-Jensen cultures. Both the H37Rv and H37Ra cells
showed a positive reaction.

The above experiments were repeated several times with the same results. This shows that if the methanol wash is omitted both virulent and avirulent tubercle bacilli give a positive test.

A few cc's of Dubos medium was added to a tube with a mixture of 5 cc of the barbiturate buffer and 1 cc of the neutral red. Instantaneously a red tinge resulted in the originally amber solution.

To a clump of H37Ra cells which had undergone the neutral red test with a negative reaction was added a cc of Dubos medium. The cells turned pink.

* The strains of M. tuberculosis mainly used throughout this dissertation were H37Ra, the avirulent variant of the human strain, H37; and H37Rv the virulent variant. They were obtained from Dr. W. Steenken Jr., Trudeau Laboratory, New York. (Steenken and Gardner 1946)
To Determine if Distilled Water Could be Used in Place of the 50% Methanol

Two weeks old cultures of H37Rv and H37Ra in Dubos medium were washed twice with 5 cc of distilled water, with one hour incubation at 37°C for each wash. Five cc of the barbiturate buffer and one cc of the neutral red solution were added to each tube and the readings made after one hour at room temperature. Both the H37Rv and the H37Ra cells had turned a red color.

The same procedure as above was used on growth from solid Löwenstein-Jensén cultures of H37Rv and H37Ra. With a wire loop a clump each of H37Rv and H37Ra growth was placed in separate tubes and washed twice with distilled water with 1 hour incubation at 37°C for each wash. Five cc of the barbiturate buffer and 1 cc of the neutral red solution was then pipetted into each tube. After one hour at room temperature both the H37Rv and H37Ra cells showed a pink positive reaction.

The results of these experiments in table form:

<table>
<thead>
<tr>
<th>Liquid cultures</th>
<th>Color reaction</th>
<th>Solid cultures</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>red</td>
<td>H37Rv</td>
<td>pink</td>
</tr>
<tr>
<td>H37Ra</td>
<td>red</td>
<td>H37Ra</td>
<td>pink</td>
</tr>
</tbody>
</table>

Distilled water apparently can not be substituted for the methanol.
Varying the Concentration of the Methanol
in the Wash

One clump of H37Rv from a 3 weeks old Loewenstein-Jensen slant was placed in each of 5 tubes. In another series of 5 tubes was similarly placed one clump of H37Ra cells.

One tube each of H37Rv and H37Ra were washed twice with 30% methanol. The second set of H37Rv and H37Ra was washed twice with 50% methanol, a third set with 60% methanol, a fourth set with 70% methanol and fifth set with 95% methanol. All washings were done at 37°C for the standard 2 hours. Into each of these five pairs of tubes with the washed organisms was then pipetted 5 cc of the barbiturate buffer and 1 cc of the neutral red. The tubes were kept at room temperature and readings made every 15 minutes up to 1 hour.

Effect of Washing with Different Concentration of Methanol

<table>
<thead>
<tr>
<th>Time in Minutes</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv 30%</td>
<td>M.red</td>
<td>M.red</td>
<td>M.red</td>
<td>M.red</td>
</tr>
<tr>
<td>H37Ra 30%</td>
<td>c.</td>
<td>c.</td>
<td>c.</td>
<td>c.</td>
</tr>
<tr>
<td>H37Rv 50%</td>
<td>P.red</td>
<td>M.red</td>
<td>M.red</td>
<td>M.red</td>
</tr>
<tr>
<td>H37Ra 50%</td>
<td>c.</td>
<td>c.</td>
<td>c.</td>
<td>c.</td>
</tr>
<tr>
<td>H37Rv 60%</td>
<td>P.red</td>
<td>P.red</td>
<td>M.red</td>
<td>M.red</td>
</tr>
<tr>
<td>H37Ra 60%</td>
<td>c.</td>
<td>c.</td>
<td>c.</td>
<td>c.</td>
</tr>
<tr>
<td>H37Rv 70%</td>
<td>P.red</td>
<td>P.red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>H37Ra 70%</td>
<td>c.</td>
<td>c.</td>
<td>c.</td>
<td>c.</td>
</tr>
<tr>
<td>H37Rv 95%</td>
<td>P.red</td>
<td>P.red</td>
<td>P.red</td>
<td>P.red</td>
</tr>
<tr>
<td>H37Ra 95%</td>
<td>c.</td>
<td>c.</td>
<td>c.</td>
<td>c.</td>
</tr>
</tbody>
</table>

C. means colorless
M.red means maroon red
P.red means pink red
This experiment shows that equally good results are obtained with methanol in concentration from 30 to 95%.

**Effect of Washing Cells With Various Solvents**

Various solvents were used in place of 50% methanol to prewash the cells. In all cases two washings at 37°C were used prior to subjecting the H37Rv and H37Ra cells from 3 weeks old Loewenstein-Jensen cultures to the buffer and neutral red. After the solvents were discarded the tubes containing the solvent treated cells were left to stand overnight to evaporate off any remaining solvent. Results were read one hour after the cells were placed in the buffer-neutral red mixture.

**Results:**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Cells</th>
<th>Neutral red reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>isoamyl alcohol</td>
<td>H37Rv</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>white</td>
</tr>
<tr>
<td>butyl alcohol</td>
<td>H37Rv</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>white</td>
</tr>
<tr>
<td>isopropyl alcohol</td>
<td>H37Rv</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>white</td>
</tr>
<tr>
<td>chloroform</td>
<td>H37Rv</td>
<td>pinkish white</td>
</tr>
<tr>
<td>carbon tetrachloride</td>
<td>H37Rv</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>white</td>
</tr>
<tr>
<td>acetone</td>
<td>H37Rv</td>
<td>pink white</td>
</tr>
<tr>
<td>ether</td>
<td>H37Rv</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>white</td>
</tr>
</tbody>
</table>
pretreatment | Cella | Neutral red reaction
--- | --- | ---
pentane | H37Rv | red
      | H37Ra | white
benzene | H37Rv | red
      | H37Ra | white
petroleum ether | H37Rv | red
      | H37Ra | white
50% methanol control | H37Rv | red
      | H37Ra | white

With the exception of ether, acetone and chloroform all the chemicals tested could satisfactorily replace methanol.

Effect of Varying the Concentration of Buffer and Neutral Red

a) Three different concentrations of neutral red were used with H37Rv and H37Ra cells from 3 weeks old Loewenstein-Jensen cultures in the standard test procedure.

<table>
<thead>
<tr>
<th>Concentration of Neutral red</th>
<th>H37Rv</th>
<th>H37Ra</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005%</td>
<td>lavender</td>
<td>white</td>
</tr>
<tr>
<td>0.025%</td>
<td>pink</td>
<td>pink</td>
</tr>
<tr>
<td>0.05%</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>0.01% (control)</td>
<td>pink</td>
<td>white</td>
</tr>
</tbody>
</table>

b) Two different concentrations of barbiturate buffer were used with H37Rv cells and H37Ra cells from 3 weeks old Loewenstein-Jensen cultures in the standard test procedure.
Concentration of Buffer

<table>
<thead>
<tr>
<th>Cells</th>
<th>0.5% barbiturate</th>
<th>2% barbiturate</th>
<th>1% barbiturate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5% NaCl</td>
<td>10% NaCl</td>
<td>5% NaCl (control)</td>
</tr>
<tr>
<td>H37Rv</td>
<td>pink</td>
<td>lavender</td>
<td>pink</td>
</tr>
<tr>
<td>H37Ra</td>
<td>pink</td>
<td>white</td>
<td>white</td>
</tr>
</tbody>
</table>

If the neutral red concentration is increased much above the standard 0.01% the H37Ra cells become colored and the test is not differential. If the buffer concentration is reduced much below the standard the H37Ra cells become colored.

Neutral Red Test at Different pH's

The different buffer solutions were made according to Clark and Lubs (Hawk and Bergeim, Practical Physiological Chemistry, page 24). Three weeks old H37Rv and H37Ra cells from Loewenstein-Jensen cultures were used. Readings were made one hour after the 50% methanol prewashed cells were placed in the neutral red-buffer solution at room temperature.

<table>
<thead>
<tr>
<th>pH of buffer</th>
<th>H37Rv</th>
<th>H37Ra</th>
<th>pH of buffer</th>
<th>H37Rv</th>
<th>H37Ra</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>red</td>
<td>red</td>
<td>9.0</td>
<td>red</td>
<td>pink</td>
</tr>
<tr>
<td>7.0</td>
<td>red</td>
<td>red</td>
<td>9.4</td>
<td>red</td>
<td>white</td>
</tr>
<tr>
<td>7.4</td>
<td>red</td>
<td>red</td>
<td>9.6</td>
<td>red</td>
<td>white</td>
</tr>
<tr>
<td>7.8</td>
<td>red</td>
<td>red</td>
<td>9.8</td>
<td>red</td>
<td>white</td>
</tr>
<tr>
<td>8.2</td>
<td>red</td>
<td>red</td>
<td>10.0</td>
<td>red</td>
<td>white</td>
</tr>
<tr>
<td>8.6</td>
<td>red</td>
<td>red</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
For the test to be differential the pH of the buffer must be 9.4 or greater.

Detection of Dye Absorption by the H37Rv and H37Ra Cells by the Use of Weak Acid Solutions

Four (three weeks old Dubos medium) cultures each of H37Rv and H37Ra were spun down and the standard neutral red test performed. The results of the test were recorded after one hour. Then one tube each of H37Rv and H37Ra were treated with 3 cc of 4 respective acids. After the acid treatment the acids were neutralized by adding 0.1N NaOH to the cells, after which the cells were reacidified with a new supply of the respective acids.

Results:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cells</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral red test</td>
<td>H37Rv</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Addition of 0.1 M acid:</td>
<td>KH₂PO₄</td>
<td>citric</td>
<td>acetic</td>
<td>lactic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H37Rv</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>Addition of 0.1N NaOH</td>
<td>H37Rv</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Addition of 0.1M acid:</td>
<td>KH₂PO₄</td>
<td>citric</td>
<td>acetic</td>
<td>lactic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H37Rv</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
</tbody>
</table>

It is apparent from these experiments that both the H37Ra and H37Rv cells absorb the neutral red dye.
Influence of Temperature on the 
Neutral Red Test

Using three week old Loewenstein-Jensen cultures of H37Rv and H37Ra both the modified* and the standard neutral red test were performed at 5 different temperatures, namely 25°C, 30°C, 37°C, 8°C, and 56°C.

In the standard test the alcohol pretreatment was performed at 37°C. After the cells were immersed in the buffer neutral red one pair each of the tubes containing the H37Rv and the H37Ra cells respectively were placed at one of the 5 different temperatures for one hour.

Results:

Modified neutral red test cultures (3 weeks old)
Loewenstein-Jensen

<table>
<thead>
<tr>
<th>H37Rv cells</th>
<th></th>
<th>25°C</th>
<th>30</th>
<th>37</th>
<th>8</th>
<th>56**</th>
</tr>
</thead>
<tbody>
<tr>
<td>time</td>
<td></td>
<td>10 min</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 min</td>
<td>pinkish</td>
<td>lavender</td>
<td>lavender</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 min</td>
<td>pink</td>
<td>pink-red</td>
<td>pink-red</td>
<td>white</td>
</tr>
<tr>
<td>acidification with 0.1NHCl</td>
<td></td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>H37Ra cells</th>
<th></th>
<th>10 min</th>
<th>white</th>
<th>white</th>
<th>white</th>
<th>white</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30 min</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 min</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
</tbody>
</table>
acidification with 0.1N HCl  red  red  red  red  red

* The modified neutral red test will be discussed in detail in later experiments. The two 50% methanol washes are eliminated and the cells added directly to a solution containing 1 cc of 50% methanol, 1 cc of the barbiturate buffer and 1 cc of the neutral red.

** Supernatant from H37Rv and H37Ra that had undergone modified neutral red test at 56 degrees centigrade were poured out and a second modified neutral red test were done on these same cells but now at room temperature.

Results: H37Rv  ...  pink
H37Ra  ...  white

Results:

Standard neutral red test
Loewenstein-Jensen cultures (3 weeks old)

H37Rv cells

<table>
<thead>
<tr>
<th>time</th>
<th>room</th>
<th>30</th>
<th>37</th>
<th>8</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td>lavender</td>
<td>lavender</td>
<td>pink</td>
<td>pinkish</td>
<td>pinkish</td>
</tr>
<tr>
<td>30 min</td>
<td>lavender</td>
<td>lavender</td>
<td>pink</td>
<td>pinkish</td>
<td>pinkish</td>
</tr>
<tr>
<td>60 min</td>
<td>lavender-red</td>
<td>red</td>
<td>red</td>
<td>pinkish*</td>
<td>pinkish</td>
</tr>
</tbody>
</table>

acidification with 0.1N HCl  red  red  red  red  red

H37Ra cells

<table>
<thead>
<tr>
<th>time</th>
<th>room</th>
<th>30</th>
<th>37</th>
<th>8</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td>pinkish</td>
<td>pinkish</td>
<td>pinkish</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>30 min</td>
<td>pinkish</td>
<td>pinkish</td>
<td>pinkish</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>60 min</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>pinkish*</td>
<td>white</td>
</tr>
</tbody>
</table>
acidification
with 0.1N HCl  red red red red red red

* Both H37Rv and H37Ra are pinkish at icebox temperature (8 degrees centigrade). However after the one hour in the icebox the H37Ra tubes were taken out and put into the 56°C water bath and in ten minutes the H37Ra cells showed a negative reaction. Following the same procedure the H37Rv cells were pink after 10 minutes.

With both standard and modified tests temperatures much above 37°C are unfavorable and the H37Rv cells remain uncolored. The same is true at very low temperatures.

Relative Amount of Dye Absorbed by H37Rv and H37Ra

The vacuum dried cells used in this study were from 4 weeks old Loewenstein-Jensen cultures. Into each of five 50 ml beakers was placed 0.3 grams of H37Ra cells and into each of a second set of 50 ml beakers was placed 0.3 grams of H37Rv cells. Into each of these 10 beakers was then placed 15 ml of the barbiturate buffer and 3 ml of the neutral red. After 2 hours at room temperature 16 ml of the buffer-neutral red mixture was pipetted out. Eight ml of absolute methanol was then pipetted into each of the 10 beakers. After 2 hours 8 ml of the mixture in each of the 10 beakers was pipetted into separate clean tubes and each acidified with six drops of 1N HCl. Each of the 10 tubes was then read on the Klett Summerson Colorimeter with a blue filter. Complete extraction of the dye from the cells was checked for by
adding acid to the cells. No red color resulted.

Results:

<table>
<thead>
<tr>
<th>Beaker No.</th>
<th>Colorimeter readings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H37Ra</td>
</tr>
<tr>
<td>1</td>
<td>107</td>
</tr>
<tr>
<td>2</td>
<td>97</td>
</tr>
<tr>
<td>3</td>
<td>110</td>
</tr>
<tr>
<td>4</td>
<td>106</td>
</tr>
<tr>
<td>5</td>
<td>105</td>
</tr>
</tbody>
</table>

Average ... 105

Average ... 91

std. dev. $\sqrt{\frac{54}{5}} = \sqrt{23.5} = 4.95$

std. dev. $\sqrt{\frac{84}{4}} = \sqrt{21} = 4.6$

S. E. diff. $\sqrt{\frac{(4.35)^2 + (4.6)^2}{4}} = \frac{9}{11.1} = 3.34$

$\frac{105 - 91}{3.34} = 4.1$

There seems to be a significantly greater amount of dye absorbed by the H37Ra cells.

Neutral Red Test Performed on Cells Killed

With Various Chemical Agents

Into duplicate sets of 4 tubes were placed respectively 5 cc of 5% phenol, 5 cc of absolute ethanol, 5 cc of 0.27 mercuric chloride, 5 cc of ether. Into each set of tubes were placed a clump of prewashed cells from three weeks old Loewenstein-Jensen cultures. The cells were allowed to remain in the chemicals for 30 minutes after which the chemical agents were discarded and the cells rinsed twice with 5 cc of distilled water. Five cc of the buffer and one cc of the neutral red were then pipetted into each
tube. After one hour at room temperature the results were read. As a water control the H37Rv and H37Ra cells were immersed in distilled water for 30 minutes. As a control of killing effect a loopful of the aqueous suspension of the cells after treatment with the respective agents were smeared onto Loewenstein-Jensen slants and incubated for six weeks. No observable growth were seen in all cases except in the controls.

<table>
<thead>
<tr>
<th>Cells</th>
<th>phenol</th>
<th>ethanol</th>
<th>mercuric chloride</th>
<th>ether</th>
<th>water</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>H37Ra</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
</tbody>
</table>

Cells killed by various chemical agents apparently react much the same as live cells.

**Effect of Treatment of Aqueous Suspensions of H37Rv and H37Ra Cells with Heat**

Three weeks old H37Rv and H37Ra cells from Loewenstein-Jensen cultures that had been pretreated with two 50% methanol washings were used.

One clump of H37Rv cells was placed in each of a series of tubes containing 5 cc of distilled water. One clump of H37Ra cells was likewise placed in each of another series of five tubes. One tube each from the two series of tubes were then placed in a beaker of boiling water for different lengths of time. After each tube was taken out, after its designated time, it was cooled immediately in a beaker of ice water. The water in each tube was
decanted and five cc of the buffer and 1 cc of the neutral red was 
than pipetted into each tube. Readings were made after 10, 30, 
60, and 120 minutes. As controls H37Rv and H37Ra cells were plac-
ed in tubes with 5 cc of water for identical intervals of time but 
standing at room temperature instead of in boiling water.

Results:

<table>
<thead>
<tr>
<th>Cells</th>
<th>Length of Heat Treatment</th>
<th>Reaction at following time intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 mins</td>
</tr>
<tr>
<td>H37Rv</td>
<td>5 mins</td>
<td>lavender</td>
</tr>
<tr>
<td></td>
<td>15 mins</td>
<td>lavender</td>
</tr>
<tr>
<td></td>
<td>30 mins</td>
<td>lavender</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>lavender</td>
</tr>
<tr>
<td></td>
<td>2 hours</td>
<td>lavender</td>
</tr>
<tr>
<td>H37Ra</td>
<td>5 mins</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>15 mins</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>30 mins</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>2 hours</td>
<td>white</td>
</tr>
<tr>
<td>Controls</td>
<td>H37Rv</td>
<td>5 mins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 mins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 mins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 hour</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 hours</td>
</tr>
</tbody>
</table>
Length of Reaction at following time intervals

<table>
<thead>
<tr>
<th>Cells</th>
<th>Heat Treatment</th>
<th>10 mins</th>
<th>30 mins</th>
<th>1 hour</th>
<th>2 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Ra</td>
<td>5 mins</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>15 mins</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>30 mins</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>1 hour</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>2 hours</td>
<td>pinkish</td>
<td>pinkish</td>
<td>white</td>
<td>white</td>
</tr>
</tbody>
</table>

Immersion in boiling water for more than 15 minutes causes a change in the H37Rv cells with resulting failure to give the typical neutral red reaction. The H37Ra cells react neutral red negative like the controls.

**Effect of Treatment of "clumps" of H37Rv and H37Ra Cells with Heat Prior to the Neutral Red Test**

The procedure followed in this experiment is similar to the previous experiment except that here the cells are placed in dry tubes with no water added.

**Results:**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Heat Treatment</th>
<th>10 mins</th>
<th>30 mins</th>
<th>1 hour</th>
<th>2 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>5 mins</td>
<td>lavender</td>
<td>lavender</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>15 mins</td>
<td>lavender</td>
<td>lavender</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>30 mins</td>
<td>lavender</td>
<td>pink</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>60 mins</td>
<td>pinkish</td>
<td>pink</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td>120 mins</td>
<td>lavender</td>
<td>lavender</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>Cells</td>
<td>Treatment</td>
<td>Length of Heat</td>
<td>Reaction after following time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>----------------</td>
<td>------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 mins</td>
<td>30 mins</td>
<td>1 hour</td>
</tr>
<tr>
<td>H37Ra</td>
<td>5 mins</td>
<td>pinkish</td>
<td>pink</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>15 mins</td>
<td>pinkish</td>
<td>pink</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>30 mins</td>
<td>pinkish</td>
<td>pink</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>60 mins</td>
<td>pinkish</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>120 mins</td>
<td>pinkish</td>
<td>pink</td>
<td>white</td>
<td>white</td>
</tr>
</tbody>
</table>

Using the same heat treatment for the same length of time the results obtained with the modified neutral red test instead of the standard neutral red test were essentially the same as above except that at no time did the H37Ra cells show any pinkish or pink color.

Heating of clumps of tubercle bacilli without addition of water apparently does not modify their reactions to the neutral red test, whether standard or modified.

**Effect of Treating H37Rv and H37Ra Cells with HCl and NaOH Prior to the Neutral Red Test**

a) One loopful of 50% methanol prewashed H37Rv cells from 3 weeks old Loewenstein-Jensen slants was suspended in each of two tubes containing 5 cc of 0.01N NaOH. Similarly, one loopful of 50% methanol prewashed H37Ra cells from 3 weeks old Loewenstein-Jensen slants was suspended in each of two tubes containing 5 cc of 0.01N NaOH. One of the H37Rv and one of the H37Ra tubes were placed in a boiling water bath for 15 mins. The other H37Rv
and H37Ra tubes were allowed to stand at room temperature for 15 mins. These pretreated cells were then subjected to the neutral red test after which dye absorption was tested by acidification of the cells with 3 cc of 0.1N HCl.

b) The same procedure as in a) was followed except here the cells were treated with 0.1N HCl for 15 minutes at room temperatures and at boiling water bath temperature.

As water controls one loopful of H37Rv and H37Ra was placed respectively in tow tubes containing 5 cc of distilled water and put into the boiling water bath for 15 minutes. A duplicate set of H37Rv and H37Ra cells in distilled water was allowed to stand at room temperature for 15 minutes. After the water was discarded the neutral red test was performed on the cells and absorption of dye was checked for by acidification with HCl.

Results:

<table>
<thead>
<tr>
<th>Cells</th>
<th>Pretreatment</th>
<th>Neutral Red Test</th>
<th>Dye Absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>0.01N NaOH in boiling water bath for 15 mins</td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>H37Ra</td>
<td></td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>H37Rv</td>
<td>0.01N NaOH at room temp. for 15 mins</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>H37Ra</td>
<td></td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>H37Rv</td>
<td>0.01N HCl in boiling water bath for 15 mins</td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>H37Ra</td>
<td></td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>H37Rv</td>
<td>0.01N HCl at room temp. for 15 mins</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>H37Ra</td>
<td></td>
<td>negative</td>
<td>positive</td>
</tr>
</tbody>
</table>
Dilute acid and alkali at room temperature has no detectable effect. At temperatures of 100°C for 15 minutes the H37Rv cells no longer react neutral red positive. Boiling water alone brings about this change but more slowly as brought out in a previous experiment.

**Effect of Treating H37Rv and H37Ra Cells With Proteolytic Enzymes Prior to the Neutral Red Test**

The enzymes were dissolved in distilled water and adjusted to the optimum pH with NaOH or HCl. The Bechman pH meter was used to check the pH's. Using 3 weeks old cells from Loewenstein-Jensen slants, prewashed twice with 50% ethanol, several loopfuls of respective cells were suspended separately into tubes containing 5 cc of the respective enzymes for 2 hours at 37°C. After this treatment the enzymes solutions were discarded and the cells rinsed twice with distilled water. The neutral red test was then performed on these cells.

The enzyme solutions were adjusted to the following pH values: pepsin 1.2, trypsin 7.8, and papain 7.0.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Pretreatment</th>
<th>Neutral Red Test</th>
<th>Dye Absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>water control</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td></td>
<td>boiling water bath</td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>H37Ra</td>
<td>water control</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td></td>
<td>room temperature</td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>Enzyme Pretreatment</td>
<td>Cells</td>
<td>Neutral red Reaction after 1 hour</td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>------------</td>
<td>-----------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Papain 1%</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.0</td>
<td>H37Rv</td>
<td>pink</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>white</td>
<td></td>
</tr>
<tr>
<td>controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water</td>
<td>H37Rv</td>
<td>pink</td>
<td></td>
</tr>
<tr>
<td>pH 7.0</td>
<td>H37Ra</td>
<td>white</td>
<td></td>
</tr>
<tr>
<td><strong>Pepsin 1%</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 1.2</td>
<td>H37Rv</td>
<td>red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>pink and white flecks</td>
<td></td>
</tr>
<tr>
<td>controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 1.2 water</td>
<td>H37Rv</td>
<td>red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>pink and white flecks</td>
<td></td>
</tr>
<tr>
<td><strong>Trypsin 1%</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.8</td>
<td>H37Rv</td>
<td>pink red</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>white</td>
<td></td>
</tr>
<tr>
<td>controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water</td>
<td>H37Rv</td>
<td>pink red</td>
<td></td>
</tr>
<tr>
<td>pH 7.8</td>
<td>H37Ra</td>
<td>white</td>
<td></td>
</tr>
</tbody>
</table>

Treatment with the 3 proteolytic enzymes for two hours at 37°C has no significant effect on the results of the neutral red test. It can be seen that treatment with pepsin seems to give the H37Ra cells a slightly positive reaction but this was not due to the pepsin itself but due to the pretreatment with a pH of 1.2 aqueous solution as the pH control showed the H37Ra with a slight positive reaction also.
**Effect of Formaldehyde on the Neutral Red Test**

a) Two clumps of H37Rv and H37Ra cells from 3 weeks old Loewenstein-Jensen slants were prewashed with 50% methanol. One clump each of H37Rv and H37Ra were treated with 5 cc of 10% commercial formaldehyde for two hours and one clump each of H37Rv and H37Ra was treated with 5 cc of 40% commercial formaldehyde for two hours. Formaldehyde from both sets were then discarded and the cells rinsed twice with distilled water before subjecting them to the buffer and the neutral red. A control was established by substituting the formaldehyde treatment with two hours water treatment.

b) Here the formaldehyde was incorporated into the neutral red-buffer cell system itself. This was done by making up a double strength of the buffer and then diluting with an equal volume of commercial formaldehyde in one case and in another case and in another case with 50% commercial formaldehyde. One set of 50% methanol prewashed H37Rv and H37Ra cells was then subjected to 5 cc of the 50% formaldehyde-5% NaCl-1% barbiturate buffer and 1 cc of 0.01% neutral red for one hour. Another set of 50% methanol prewashed H37Rv and H37Ra cells was subjected to 5 cc of the 25% formaldehyde-5% NaCl-1% barbiturate buffer and 1 cc of the 0.01% neutral red for one hour.

In both cases, whether the cells were pretreated with the formaldehyde or the formaldehyde incorporated into the buffer-neutral red cell system there was no detectable effect on the
**Extraction of the Dye from Neutral Red Stained Cells**

a) Three weeks old cultures of H37Rv and H37Ra in Dubos medium were used. After the cells were spun down and the media decanted the neutral red test was performed on the H37Rv and H37Ra cells contained in 5 duplicate sets of tubes. After one hour, when the H37Rv cells have shown its characteristic red color, the supernatant of all 5 duplicate sets of H37Rv and H37Ra were discarded and one set of each of the 5 sets were treated with a specific concentration of methanol in 5 cc amounts. The H37Ra cells were first acidified with 0.1N HCl to make the dye on the cells visible. Five different concentrations of methanol ranging from 30% to 95% were used. Disappearance of color was checked after 15 minutes, 1 hour and after 24 hours. After the color had totally disappeared from the cells the supernatant alcohol was decanted into clean tubes and 3 cc of 0.1N HCl added. The cells remaining in the tubes were subjected to a second neutral red test. This last step was performed only on cells subjected to the 70 and 95% methanol extraction. The cells that had been subjected to other concentrations of methanol (30, 50, 60%) were subjected to acid treatment after the supernatant alcohol was decanted off.

* Commercial formaldehyde - 37% formaldehyde solutions
### Results:

**H37Rv cells**

<table>
<thead>
<tr>
<th>Tube</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral red reaction</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>Alcohol extraction</td>
<td>30%</td>
<td>50%</td>
<td>60%</td>
<td>70%</td>
<td>95%</td>
</tr>
<tr>
<td>15 minutes</td>
<td>pink-red</td>
<td>pinkish</td>
<td>pinkish</td>
<td>pinkish</td>
<td>white</td>
</tr>
<tr>
<td>1 hour</td>
<td>pink-red</td>
<td>pinkish</td>
<td>pinkish</td>
<td>pinkish</td>
<td>white</td>
</tr>
<tr>
<td>24 hours</td>
<td>pink</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Acidification of decanted alcohol</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>Cells acidified</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Neutral red test repeated</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
</tbody>
</table>

**H37Ra cells**

<table>
<thead>
<tr>
<th>Tube</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral red reaction</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Acidification with 0.1N HCl</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>Alcohol extraction</td>
<td>30%</td>
<td>50%</td>
<td>60%</td>
<td>70%</td>
<td>95%</td>
</tr>
<tr>
<td>15 minutes</td>
<td>pink-red</td>
<td>pinkish</td>
<td>pinkish</td>
<td>pinkish</td>
<td>white</td>
</tr>
<tr>
<td>1 hour</td>
<td>pink-red</td>
<td>pinkish</td>
<td>pinkish</td>
<td>pinkish</td>
<td>white</td>
</tr>
<tr>
<td>24 hours</td>
<td>pink</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Acidification of decanted alcohol</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>Cells acidified</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
</tbody>
</table>
b) Using the same procedure as in part a) the extracting power of ethanol, butyl alcohol, isopropyl alcohol, chloroform, ether, carbon tetrachloride and acetone was tested. As in part a) the H37Ra cells were treated with a weak acid solution to give them a red color before testing for extraction of the dye. In all cases disappearance of color was checked after an hour and in all cases the cells were found to be colorless. Extraction of the dye was checked by adding one cc of 0.01N HCl to the solvents after they were poured off into separate clean tubes. A second neutral red test performed on the cells that had been treated with the solvents showed in all cases a positive test for the H37Rv cells and a negative test for the H37Ra cells.

The neutral red absorbed by the H37Rv and H37Ra cells can be extracted with methanol. The higher the concentration of methanol used the faster the extraction of the dye. There is no differential rate of extraction of the dye in the H37Rv cells and H37Ra cells. Other agents can also be used to extract the dye.

Apparently extraction of the dye does not hinder the cells from giving a second neutral red test with equally good differential color reaction.
Differential Decolorization with NaOH of Neutral Red Stained H37Rv, H37Ra Cells

a) After the neutral red test was performed on H37Rv and H37Ra cells the supernatant solution was discarded and the cells rinsed twice with distilled water. Five cc of 0.1N acetic acid was added to both and in a minute or so the H37Ra cells turned red. Thus at this point both the H37Rv and H37Ra cells were red. The acid was poured off and 5 cc of 0.1N NaOH was added to the cells. The H37Ra cells were decolorized within one minute whereas the H37Rv cells were still red after one hour.

b) Several clumps each of H37Rv and H37Ra growth from Loewenstein-Jensen slants were suspended for one hour in separate tubes containing 5 cc of 0.01% neutral red solution. The cells were then subjected to the decolorization process with 5 cc of 0.1N NaOH. The H37Ra cells were decolorized in 2 minutes whereas the H37Rv cells were still red after one hour. At two hours the H37Rv cells were pink and at 5 hours they were orange.

c) Here the 50% methanol prewashed H37Rv and H37Ra cells were suspended in the buffer-neutral red mixture for 10 minutes instead of one hour as in part a). The cells were subjected first to the acid and then the alkali treatment. The H37Ra cells were colorless after 1 minute and the H37Rv cells were decolorized in 15 minutes.

d) Here the H37Rv and H37Ra cells were subjected to one hour treatment with an 0.01% neutral red solution and the effect
determined of decolorizing with 5 cc of buffers ranging in pH from 7 to 10.

<table>
<thead>
<tr>
<th>buffer pH#</th>
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<tr>
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</tr>
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<td>red + yellow</td>
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</table>

* Buffers prepared according to Clark and Lubs, Practical Physiological Chemistry by Bergeim. pH checked with Beckman pH meter.

e) Here the H37Rv and H37Ra cells were subjected to one hour treatment with an 0.01% neutral red solution as in part d) but here the effect of extraction with 5 cc of different concentrations of methanol ranging from 10 to 90% were tested.

<table>
<thead>
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<th>% Methanol</th>
<th>cells</th>
<th>1 hour</th>
<th>16 hours</th>
<th>48 hours</th>
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</thead>
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<tr>
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<td>reddish</td>
</tr>
<tr>
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<td>red</td>
<td>reddish</td>
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</tr>
<tr>
<td></td>
<td>H37Ra</td>
<td>red</td>
<td>reddish</td>
<td>reddish</td>
</tr>
<tr>
<td>% Methanol</td>
<td>Methanol cells</td>
<td>1 hour</td>
<td>16 hours</td>
<td>48 hours</td>
</tr>
<tr>
<td>------------</td>
<td>----------------</td>
<td>--------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td><strong>H37Rv</strong></td>
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<td><strong>reddish</strong></td>
<td><strong>reddish</strong></td>
</tr>
<tr>
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<td><strong>reddish</strong></td>
</tr>
<tr>
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<td><strong>pink</strong></td>
<td><strong>lavender</strong></td>
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<tr>
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<td><strong>pinkish</strong></td>
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<tr>
<td>60</td>
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</tr>
</tbody>
</table>

Whether the cells had undergone the neutral red test or treated with a 0.01% neutral red for one hour there is a differential decolorization with NaOH and buffers ranging from pH 9 - pH 10 between the H37Rv and H37Ra cells. As can be expected the shorter the time of exposure of the cells to the dye, the faster is the decolorization with alkali but differential decolorization between the H37Rv and H37Ra cells remain. There is no evidence of differential extractibility of the dye between the H37Rv and H37Ra cells.

**Extraction of Neutral Red Factor**

Thirty petri plates each containing 15 ml of Loewenstein-Jensen medium and a cellophane sheet covering the entire surface of the media were seeded with H37Rv cells and incubated at 37°C. After
4 weeks the plates were harvested by removing the cellophane sheets with the bacteria and washing off the growth from the cellophane by immersing in a beaker containing 50% methanol. After an hour the methanol was decanted and replaced with fresh 50% methanol for another hour. The supernatant methanol was then poured off and the bacteria dried in a vacuum dessicator. The dried cells (2.33 grams were then extracted with ether (anesthetic grade) with a soxhlet apparatus for 24 hours. The cells were then tested and found to be neutral red negative but still acid fast positive. The ether was then distilled off at 40°C leaving residue which consisted of a brownish oily substance along with a waxy colorless substance. A loopful of this mixture was transferred to a tube containing 5 cc of buffer and 1 cc of neutral red. In about 3 minutes it was red and became very intense red within an hour.

A loopful of the ether extracted H37Rv cells, which were found previously to be neutral red negative, was immersed in this oily-waxlike mixture from the ether extraction and placed into the buffer neutral red solution. They now gave a positive neutral red test. In the same manner H37Ra cells were coated with the extract from the H37Rv cells and was found to give a neutral red positive reaction.

Using the same procedure 5.7 grams (dried weight) of H37Ra cells was extracted with ether. A brownish oily residue was obtained after the ether extraction which did not give a positive neutral red test.
Several loopfuls of this lipid like substance extracted from H37Rv cells were dissolved in several cc of ether. One cc of this solution was added to ether extracted H37Rv cells and the ether subsequently evaporated off by vacuum. The neutral red test was then performed on these H37Rv cells. They showed a positive neutral red test where previously they were negative.

One loopful of the same ether solution was added to H37Ra cells (both untreated and extracted with ether) and the ether evaporated off. The neutral red test performed on them showed a positive reaction.

A substance which gives a neutral red positive test can be extracted from H37Rv cells but not from H37Ra cells.

* If a few cc's of the ether that was used to treat the H37Rv cells are evaporated off in a test tube, a thin film of the ether extract will surround the sides of the test tube. Though this film will be neutral red positive up to 15 minutes or so it will be decolorized or neutral red negative by an hour.

Effect on Neutral Red Test When Cells Are Treated with Sonic Vibration

Dried four weeks old 50% methanol prewashed H37Rv and H37Ra cells were used. One half gram of H37Rv cells were suspended in 25 cc of distilled water and treated with the Raytheon Sonic Oscillator at 9000 cycles/sec for a period of one hour. The resulting milky suspension was then spun down at approximately 5000 RPM.
with the Servall angle centrifuge. Smears made of the sedimented material showed them to be acid fast rods. These cells were found to be neutral red negative although dye was absorbed as determined with 0.1N HCl. A control consisting of suspending one half gram of dried 50% methanol pretreated H37Rv cells in 25 cc of distilled water for one hour and spun down at 5000 RPM was neutral red positive. The supernatant of the H37Rv cells was then spun down at 16000 RPM for an hour with the Servall Vacuum Centrifuge and were found to consist of a mixture of amorphous non acid fast masses and non acid fast gramules of approximately one half micron in size. This material was also neutral red negative and again the dye was absorbed as determined by 0.1N HCl. The supernatant was treated with 100 cc of ether for 24 hours. The ether was separated off with a separatory funnel and evaporated off by vacuums. No neutral red positive substance was obtained. However, the acid fast cells that were spun down at 5000 RPM did produce a substance that was neutral red positive. This substance was extracted out with ether.

Following the same procedure H37Ra cells were treated with the sonic oscillator. They remained neutral red negative.

b) The procedure here is similar to that in part a) except that the neutral red test was first performed on one half gram of dried 50% methanol pretreated H37Rv cells. After one hour when the cells showed a pink-red color they were treated with the sonic oscillator. A pink milky suspension resulted. This was spun
down at 5000 RPM for a half hour. The sediment was pink color as was the supernatant. One loopful of the sedimented cells was placed in 5 cc of 0.1N NaOH and was decolorized immediately. One loopful of the sedimented cells placed in 5 cc of the 1% barbiturate buffer was decolorized in about 3 minutes.

The supernatant was spun down at 16000 RPM for one hour with the Servall vacuum centrifuge. The sediment was also pink color which was also decolorized when placed in 0.1N NaOH or the barbiturate buffer.

It seems that sonic vibration treatment alters permeability of the H37Rv cells such that they are more permeable to the buffer thus making them behave like the H37Ra cells under the neutral red test.
CHAPTER IV

DETERMINATION OF THE OPTIMUM PROPORTION OF
ALCOHOL-BUFFER-NEUTRAL RED IN A
MODIFIED NEUTRAL RED TEST

The possibility of eliminating the 50% methanol pretreatment was investigated by incorporating the 50% methanol with the buffer and neutral red. It was arbitrarily decided to vary the volume of the 3 constituents by 1 part by volume, 3 parts by volume, and 5 parts by volume. With this setup of 3 constituents with 3 variable parts by volume for each we end up with 27 combinations. The pH of these 27 mixtures were determined with a Beckman pH meter. The order of dispensing of the constituents was alcohol, cells, buffer and then the neutral red. Three weeks old cultures of H37Rv and H37Ra on Loewenstein-Jensen slants were used and the stock strengths of the 3 constituents were as in the standard neutral red test, namely, 50% methanol, 5% sodium chloride-1% sodium barbiturate buffer and 0.01% neutral red.

Results:
A equals H37Ra
V equals H37Rv
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<th>Methanol</th>
<th>Buffer</th>
<th>Neutral Red</th>
<th>pH</th>
<th>Cells</th>
<th>Appearance of cells after 1 hour</th>
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</thead>
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<td>pinkish</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>V</td>
<td>red</td>
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</table>
There are 9 sets of tubes which showed distinct differentiation between the H37Rv and the H37Ra cells. Although there seems to be better differentiation with the ratio 1:3:3 and 3:5:5. For reasons of economy and simplicity the 1:1:1 ratio was adopted for the modified neutral red test. In so far as determining what

<table>
<thead>
<tr>
<th>Methanol</th>
<th>Buffer</th>
<th>Neutral Red</th>
<th>pH</th>
<th>Cells</th>
<th>Appearance of cells after 1 hour</th>
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<td>5</td>
<td>5</td>
<td>9.6</td>
<td>A</td>
<td>white</td>
</tr>
</tbody>
</table>
volume of each of the 3 constituents should be used this experimental procedure is adequate but it does not tell us whether an effect shown by increasing the volume of one constituent over the other is actually due to the increase of this one constituent or due to the dilution or decrease of the concentration of the other two constituents. The following experiment proposes to give us this information.

**Effect of Varying the Concentrations of Alcohol, Buffer and Neutral Red in the Modified Neutral Red System**

It was proposed to vary the concentrations of the alcohol, buffer and neutral red accordingly: 1) 50% NaCl-1% sodium barbiturate, 0.01% neutral red; 2) three fifths the concentrations of that in case 1; 3) one fifth the concentrations of that in case 1. The unit 5 is used to denote the concentrations of the alcohol, buffer, and neutral red as stipulated in case 1. The unit 3 is used to denote the concentrations as stipulated in case 2. The unit 1 is used to denote concentrations as stipulated in case 3. Final volume of material in all cases was constant. To illustrate, to make up a system of 50% methanol, 1% buffer, 0.01% neutral red one ml each of absolute methanol, 2% buffer and 0.02% neutral red were pipetted into a tube and then made up to a final volume of 6 cc by addition of 3 ml of distilled water. To have a system where 3/5 of this concentration was used one ml each of 3/10 concentration of 50% methanol, 1% buffer and 0.01% neutral red was pipetted into the
tube and made up to final volume of 6 ml by addition of 3 ml of distilled water. As in the previous experiment the various combinations of the 3 constituents were pipetted into the tubes in the order of alcohol, cells, buffer, and neutral red.

Results:
A means H37Ra
V means H37Rv

<table>
<thead>
<tr>
<th>Unit Concentration</th>
<th>of Constituents</th>
<th>Appearance of cells after 1 hr.</th>
</tr>
</thead>
<tbody>
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<td>50% methanol</td>
<td>5% NaCl</td>
<td>0.01% Na-Barb.</td>
</tr>
<tr>
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<td>1</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>Unit Concentration of Constituents</td>
<td>Appearance of cells after 1 hr.</td>
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<tr>
<td>50% NaCl 5% Na-Barb.</td>
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<tr>
<td>0.01% pH of tube methanol 1%</td>
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</table>

It can be seen from the data that as the concentration of neutral red is increased the color reaction, whether in the H37Rv of H37Ra, becomes more intense. It may also be observed that as the concentration of the buffer is increased the color reaction, whether of H37Rv or H37Ra, is less intense. Note also that as the alcohol concentration is increased the color reaction is decreased.

In general, the ratio of equal concentrations of the 3 constituents still shows good differentiation between the H37Rv and H37Ra cells. The ratios of 3:5:5 also showed good differentiations.

**Comparative Study of the Modified Neutral Red Test**

and the **Standard Neutral Red Test**

A total of 215 cultures of tubercle bacilli, or atypical forms of tubercle bacilli, freshly isolated from patients were used to compare the efficiency of the modified neutral red test as against the standard neutral red test. Source of the cultures were as follows:

18 cultures from Mercy Hospital, Chicago

9 cultures from Saint Agnes Hospital, Fond du Lac, Wisconsin

39 cultures from University of Illinois Research Hospital, Chicago
149 cultures from Cook County Hospital Tuberculosis Ward, Chicago

In the modified neutral red test one cc of 50% methanol was pipetted into a tube followed in order by a large clump of growth from a 3 to 4 weeks old Loewenstein-Jensen slant, 1 cc of the buffer solution and 1 cc of the neutral red solution. The standard neutral red test was performed as previously described.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Growth Characteristics*</th>
<th>Standard N.R. test</th>
<th>Modified N.R. test</th>
</tr>
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<tbody>
<tr>
<td>Mercy Hospital</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.5 avir.</td>
<td>pinkish, waxy</td>
<td>neg.</td>
<td>neg.</td>
</tr>
<tr>
<td>24.16 tb</td>
<td>pinkish yellow, fast grower</td>
<td>neg.</td>
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<tr>
<td>24.18 avir.</td>
<td>orange, fast grower</td>
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<tr>
<td>24.19 H37Rv</td>
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<td>24.20 tb</td>
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<td>pos.</td>
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</tr>
<tr>
<td>24.21 tb</td>
<td>typical but fast grower</td>
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<tr>
<td>24.23 tb</td>
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<td>neg.</td>
</tr>
<tr>
<td>24.24 atyp.</td>
<td>yellow smooth</td>
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<td>neg.</td>
</tr>
<tr>
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<td>24.34</td>
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</table>

* Growth Characteristics: A cream colored, crumb-like growth which takes approximately 2 weeks to grow out is considered "typical". Any deviations from this are described accordingly.

neg. - refers to white or amber colored cells 1 hour after the neutral red test.
pos. - refers to pink or red colored cells 1 hour after the neutral red test.
doubtful - refers to pinkish colored cells 1 hour after the neutral red test.

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Saint Agnes Hospital

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University of Illinois Research Hospital

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</tr>
<tr>
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</tr>
<tr>
<td>60a'</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
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</tr>
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</tr>
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<tr>
<td>Cultures</td>
<td>Growth Characteristics</td>
<td>Standard N.R. test</td>
<td>Modified N.R. test</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------</td>
<td>--------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>66a</td>
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<td>doubtful</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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</tr>
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</tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>96d</td>
<td>smooth, fast grower</td>
<td>neg.</td>
<td>neg.</td>
</tr>
<tr>
<td>Cultures</td>
<td>Growth Characteristics</td>
<td>Standard N.R. test</td>
<td>Modified N.R. test</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------</td>
<td>-------------------</td>
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</tr>
<tr>
<td>105a</td>
<td>typical</td>
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<td>pos.</td>
</tr>
<tr>
<td>103a</td>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>pos.</td>
</tr>
<tr>
<td>109d</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
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<td>pos.</td>
</tr>
<tr>
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<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>110a</td>
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<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>111d</td>
<td>typical</td>
<td>pos.</td>
<td>neg.</td>
</tr>
<tr>
<td>120e</td>
<td>typical</td>
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<td>pos.</td>
</tr>
<tr>
<td>121a</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>121e</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>120a</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>116a</td>
<td>typical</td>
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<td>pos.</td>
</tr>
<tr>
<td>127a</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>133e</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>134e</td>
<td>typical</td>
<td>doubtful</td>
<td>neg.</td>
</tr>
<tr>
<td>134e</td>
<td>typical</td>
<td>doubtful</td>
<td>neg.</td>
</tr>
<tr>
<td>134a</td>
<td>typical</td>
<td>neg.</td>
<td>neg.</td>
</tr>
<tr>
<td>Cultures</td>
<td>Growth Characteristics</td>
<td>Standard N.R. test</td>
<td>Modified N.R. test</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------</td>
<td>-------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>134e</td>
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<td>doubtful</td>
<td>neg.</td>
</tr>
<tr>
<td>138e</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>139e</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>143a</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>143e</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>143e</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>146a</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>146e</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>147a</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>147e</td>
<td>typical</td>
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<td>pos.</td>
</tr>
<tr>
<td>149a</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>155a</td>
<td>typical</td>
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<td>pos.</td>
</tr>
<tr>
<td>155e</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>155e</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>161a</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>161a</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>162e</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td>162a</td>
<td>typical</td>
<td>pos.</td>
<td>pos.</td>
</tr>
</tbody>
</table>

**Summary**

<table>
<thead>
<tr>
<th>Source and Number of Cultures</th>
<th>Number of Cultures</th>
<th>Standard N.R. test</th>
<th>Modified N.R. test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercy Hospital 18 Cultures</td>
<td>14</td>
<td>neg.</td>
<td>neg.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>pos.</td>
<td>neg.</td>
</tr>
<tr>
<td>Source and Number of Cultures</td>
<td>Number of Cultures</td>
<td>Standard N.R. test</td>
<td>Modified N.R. test</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------------</td>
<td>--------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>University of Illinois</td>
<td>4</td>
<td>neg.</td>
<td>neg.</td>
</tr>
<tr>
<td>39 Cultures</td>
<td>33</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>pos.</td>
<td></td>
</tr>
<tr>
<td>Cook County Hospital</td>
<td>8</td>
<td>neg.</td>
<td>neg.</td>
</tr>
<tr>
<td>149 Cultures</td>
<td>135</td>
<td>pos.</td>
<td>pos.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>pos.</td>
<td>neg.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>doubtful</td>
<td>neg.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>doubtful</td>
<td>doubtful</td>
</tr>
</tbody>
</table>

As can be seen form the summary the modified and standard neutral red test failed to agree with 4 cultures. The culture from Mercy Hospital which failed to agree between the two test showed atypical growth characteristic being dark yellow in color. One of the two cultures in the University of Illinois group was also atypical in growth characteristics in that it was white and smooth. It is conceivable that such cultures where some deviation from the normal growth characteristics is present can also very well have a deviation in some other factor such as the factor responsible for the neutral red test.

In all 4 cases the standard neutral red test showed a positive reaction whereas the modified neutral red test a negative reaction which points to the possibility that the modified neutral red test may not be as sensitive as the standard method. It was also observed that, in general, the positive reaction by the standard method were more intense in color than the positive reactions by the modified neutral red test. This may be the reason for the 3 cultures in the Cook County group which gave doubtful
(pinkish) reactions by the standard neutral red test but negative reactions by the modified test.

As a whole the agreement between the two methods was very good with only 2 typical cultures positive by the standard test and negative by the modified test. Typical growth may not always indicate virulence and the modified test may still be positive with all virulent tubercle bacilli.

The Order of Adding in the 3 Constituents of the Modified Neutral Red Test

Six different sequences of adding the constituents of the modified neutral red test to the tubes were studied. Two sets of six tubes were used. In one set the cells were three weeks old Loewenstein-Jensen cultures of H37Rv and the other set the cells were three weeks old Loewenstein-Jensen cultures of H37Ra.

Results:

<table>
<thead>
<tr>
<th>Order</th>
<th>Cells</th>
<th>Reaction at 15 min</th>
<th>1 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>alcohol</td>
<td>H37Ra</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>neutral red</td>
<td>H37Rv</td>
<td>white</td>
</tr>
<tr>
<td>II</td>
<td>buffer</td>
<td>H37Ra</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>neutral red</td>
<td>H37Rv</td>
<td>pinkish</td>
</tr>
<tr>
<td>III</td>
<td>neutral red</td>
<td>H37Rv</td>
<td>pink</td>
</tr>
<tr>
<td></td>
<td>cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>alcohol</td>
<td>H37Ra</td>
<td>red</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Order</th>
<th>Cells</th>
<th>Reaction at 15 min</th>
<th>1 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>H37Ra</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>H37Rv</td>
<td>pink</td>
<td>pink</td>
</tr>
<tr>
<td>V</td>
<td>H37Ra</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>H37Rv</td>
<td>pink</td>
<td>pink</td>
</tr>
<tr>
<td>VI</td>
<td>H37Ra</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>H37Rv</td>
<td>pink</td>
<td>pink-red</td>
</tr>
</tbody>
</table>

Sequence III and sequence VI seems to give the most definite color differentiation between the H37Rv and H37Ra cells but with sequence III it is possible that the pink color of the H37Ra cells could be prolonged even up to the 1 hour thus giving the H37Ra cells a false positive reaction. Because of this it seems that of all the six orders studied the sixth sequence of neutral red, cells, alcohol and then buffer is the best.

Performing the Modified Neutral Red Test with a Mixture of 2 or 3 of the Chemical Solutions of the Test

<table>
<thead>
<tr>
<th>Order of Constituents</th>
<th>Cells</th>
<th>Neutral red reaction at 15 mins.</th>
<th>60 mins.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 cc of 1:1:1 mixture of methanol-buffer-neutral red.</td>
<td>H37Ra</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>H37Rv</td>
<td>pinkish</td>
<td>pinkish</td>
</tr>
<tr>
<td>Order of Constituents</td>
<td>Cells</td>
<td>Neutral red reaction at 15 mins.</td>
<td>Neutral red reaction at 60 mins</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------</td>
<td>-------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>1 cc of methanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>H37Ra</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>2 cc of 1:1 mixture of buffer-neutral red</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>H37Rv</td>
<td>pinkish</td>
<td>pinkish</td>
</tr>
<tr>
<td>1 cc neutral red</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 cc of 1:1 mixture of buffer-neutral red</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>H37Ra</td>
<td>pinkish</td>
<td>white</td>
</tr>
<tr>
<td>1 cc methanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 cc methanol-neutral red mixture (1:1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>H37Rv</td>
<td>pinkish</td>
<td>pinkish</td>
</tr>
<tr>
<td>1 cc buffer</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Except for the mixing of the methanol and buffer into one solution, less clear cut differentiation of the virulent and non-virulent cells is obtained by mixing the ingredients before addition of the cells.

**Elimination of the Need for Methanol in the Modified Neutral Red Test by Use of a Proper Buffer**

Four different variations were used. In the first
experiment the modified neutral red test with 1 cc of water in place of the alcohol and with the pH of the buffer varying from pH 1.2 to 10.0 was performed on cells that were not pretreated in any way.

In the second experiment the cells were pretreated with two distilled water washes (1 hour each wash) then the modified neutral red test with 1 cc of water in place of the alcohol was performed with the pH of the buffer varying from pH 1.2 to 10.

In the third experiment the cells were pretreated with two 50% methanol washes then the modified neutral red test with 1 cc water in place of the alcohol was performed with the pH of the buffer varying from 1.2 to 10.

In the fourth experiment the cells were pretreated with two acetone washes (1 hour each wash) then the modified neutral red test with 1 cc of distilled water in place of the alcohol was performed with the pH of the buffer varying from 1.2 to 10.0.

The control consisted of the modified neutral red test with the alcohol and buffers from pH 1.2 to 10.0. The different pH buffer solutions were prepared according to Clark and Luba (Hawk and Bergeim, Practical Physiological Chemistry, page 24).

Results:

<table>
<thead>
<tr>
<th>No Pretreatment</th>
<th>1 cc alc</th>
<th>1 cc buffer</th>
<th>1 cc water</th>
<th>1 cc buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH of buffer</td>
<td>H37Rv</td>
<td>H37Ra</td>
<td>H37Rv</td>
<td>H37Ra</td>
</tr>
<tr>
<td>1.2</td>
<td>lavender-white red</td>
<td>pinkish red</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH of buffer</td>
<td>1 cc alc</td>
<td>1 cc buffer</td>
<td>1 cc neutral red</td>
<td>1 cc water</td>
</tr>
<tr>
<td>--------------</td>
<td>---------</td>
<td>-------------</td>
<td>------------------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>H37Rv</td>
<td>H37Ra</td>
<td></td>
<td>H37Rv</td>
</tr>
<tr>
<td>2.0</td>
<td>lavender-white</td>
<td>red</td>
<td>pinkish</td>
<td>red</td>
</tr>
<tr>
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<td>pink</td>
<td>red</td>
<td>pinkish</td>
<td>red</td>
</tr>
<tr>
<td>4.0</td>
<td>pink</td>
<td>red</td>
<td>pink</td>
<td>red</td>
</tr>
<tr>
<td>5.0</td>
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<td>pink</td>
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</tr>
<tr>
<td>6.0</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>7.0</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>7.4</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>7.8</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>8.2</td>
<td>red</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>8.6</td>
<td>red</td>
<td>pink</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>9.0</td>
<td>red</td>
<td>pinkish</td>
<td>red</td>
<td>pink</td>
</tr>
<tr>
<td>9.4</td>
<td>red</td>
<td>white</td>
<td>red</td>
<td>pink</td>
</tr>
<tr>
<td>9.8</td>
<td>red</td>
<td>white</td>
<td>red</td>
<td>pinkish</td>
</tr>
<tr>
<td>10.0</td>
<td>red</td>
<td>white</td>
<td>red</td>
<td>white</td>
</tr>
</tbody>
</table>

Results:
Pretreated by 2 water washes then 1 cc water, 1 cc buffer, and 1 cc neutral red.

<table>
<thead>
<tr>
<th>pH of buffer</th>
<th>H37Rv</th>
<th>H37Ra</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>lavender-white</td>
<td>red</td>
</tr>
<tr>
<td>2.0</td>
<td>lavender-white</td>
<td>red</td>
</tr>
<tr>
<td>3.0</td>
<td>lavender-white</td>
<td>red</td>
</tr>
<tr>
<td>4.0</td>
<td>lavender-white</td>
<td>red</td>
</tr>
<tr>
<td>5.0</td>
<td>lavender-white</td>
<td>red</td>
</tr>
</tbody>
</table>
### Results:
Pretreated with two 50% methanol washes (1 hour each wash) then 1 cc water, 1 cc respective buffers, and 1 cc neutral red

<table>
<thead>
<tr>
<th>pH of buffer</th>
<th>H37Rv</th>
<th>H37Ra</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>pink-red</td>
<td>red</td>
</tr>
<tr>
<td>7.0</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>7.4</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>7.8</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>8.2</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>8.6</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>9.0</td>
<td>red</td>
<td>pink</td>
</tr>
<tr>
<td>9.4</td>
<td>red</td>
<td>pink</td>
</tr>
<tr>
<td>9.8</td>
<td>red</td>
<td>pinkish</td>
</tr>
<tr>
<td>10.0</td>
<td>red</td>
<td>white</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH of buffer</th>
<th>H37Rv</th>
<th>H37Ra</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>lavender-white</td>
<td>red</td>
</tr>
<tr>
<td>2.0</td>
<td>lavender-white</td>
<td>red</td>
</tr>
<tr>
<td>3.0</td>
<td>lavender-white</td>
<td>red</td>
</tr>
<tr>
<td>4.0</td>
<td>lavender-white</td>
<td>red</td>
</tr>
<tr>
<td>5.0</td>
<td>lavender-white</td>
<td>red</td>
</tr>
<tr>
<td>6.0</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>7.0</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>7.4</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>7.8</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>8.2</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>8.6</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>pH of buffer</td>
<td>H37Rv</td>
<td>H37Ra</td>
</tr>
<tr>
<td>--------------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>9.0</td>
<td>red</td>
<td>pink</td>
</tr>
<tr>
<td>9.4</td>
<td>red</td>
<td>pink</td>
</tr>
<tr>
<td>9.8</td>
<td>red</td>
<td>pink</td>
</tr>
<tr>
<td>10</td>
<td>red</td>
<td>pinkish</td>
</tr>
</tbody>
</table>

In general the red here is darker than with the two previous sets with no wash or with the water wash.

Results:
Pretreated with acetone wash twice (1 hour each wash then 1 cc of water, 1 cc of respective buffer and 1 cc of neutral red.

<table>
<thead>
<tr>
<th>pH of buffer</th>
<th>H37Rv</th>
<th>H37Ra</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>lavender</td>
<td>red</td>
</tr>
<tr>
<td>2.0</td>
<td>lavender</td>
<td>red</td>
</tr>
<tr>
<td>3.0</td>
<td>lavender</td>
<td>red</td>
</tr>
<tr>
<td>4.0</td>
<td>lavender</td>
<td>red</td>
</tr>
<tr>
<td>5.0</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>6.0</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>7.0</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>7.4</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>7.8</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>8.2</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>8.6</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>9.0</td>
<td>red</td>
<td>pink</td>
</tr>
<tr>
<td>9.4</td>
<td>red</td>
<td>white</td>
</tr>
<tr>
<td>9.8</td>
<td>red</td>
<td>white</td>
</tr>
<tr>
<td>10</td>
<td>pink</td>
<td>white</td>
</tr>
</tbody>
</table>
These experiments show that by increasing the pH of the buffer used in the modified neutral red test eliminating the need for the use of any alcohol in the test. This was true whether the cells were not pretreated or pretreated with water washes or acetone washes.

In general, the intensity of the red color in cells pretreated with acetone and subsequently undergoing the modified neutral red test seemed to be less than that seen in cells pretreated with methanol or water. However, this does not constitute clear cut evidence that acetone by virtue of tying-up Coo-groups decreases the intensity of the neutral red color reaction of H37Rv cells.

It should be noted that there seems to be more dye absorbed by H37Ra cells than H37Rv cells at the acid pH's of 1.2 to approximately pH 5.0.

**Modified Neutral Red Test Performed With Liquid Cultures**

Duplicate sets of 27 liquid cultures (Dubos medium) obtained originally from Cook County tuberculosis wards were used. With one set the modified neutral red test was performed. All 27 cultures when done by the standard method gave a positive neutral red reaction. All 27 cultures when done by the modified neutral red test gave a negative reaction. When the supernatant solution from this latter set was poured off and the cells acidified there was but a slight lavender color which indicated that the absorption of dye was hindered. The modified test apparently cannot be used
with cultures in Dubos medium.

**Performing the Neutral Red Test with H37Rv and H37Ra Cells Crown on Loewenstein-Jensen Culture Slants**

Three cc each of 50% methanol, barbiturate buffer and neutral red were pipetted on to a three weeks old H37Rv and a three weeks old H37Ra culture slant and allowed to stand at room temperature for one hour. Both the H37Rv and the H37Ra cells on the culture slants showed a negative reaction. Several clumps of H37Rv and H37Ra were taken out of their respective culture slants and put into separate tubes, rinsed twice with distilled water and then acidified with 2 cc of 0.1 N HCl. No red color resulted. Absorption of dye seems to be interfered with when the modified neutral red test is done on the organisms on the culture slants.

Several clumps of growth were taken from the H37Rv and H37Ra slants that had previously had the modified neutral red test done on them and put into separate clean tubes and a second modified neutral red test performed. Result showed that the H37Ra gave a negative and the H37Rv a slight pinkish reaction. This was not unexpected as it was observed that when the modified neutral red test was done cells that were taken from slants which had a lot of water of condensation, and consequently wetting the growth on the slant, the neutral red test (modified) would not be satisfactory in that the H37Rv cells gave a poor positive to negative reaction.

Several clumps of H37Rv and H37Ra growth were taken from
the culture slants that had previously had the modified neutral red test done on them and the standard neutral red test was performed. The H37Rv cells gave a positive reaction whereas the H37Ra cells a negative reaction. The 50% methanol wash apparently washed away the inhibitor from the media.

The standard neutral red test was done directly on the H37Rv and H37Ra growth remaining on the slants that had previously undergone the modified neutral red test. Both the H37Rv and H37Ra cells gave a negative reaction.

It seems that the neutral red test whether the standard or the modified can not be performed on the cells as they are in the culture slants because of interference with absorption of the dye by the organisms.

**Effect of Sodium Desoxycholate on the Neutral Red Test**

Three cultures that were neutral red negative (33605, 2418 avir, and H37Ra) and two cultures that were neutral red positive (H37Rv and FDL 5) were used. It was decided to incorporate the bile salt into the modified neutral red test in the final concentrations of 2%, 1%, 0.5% and 0.25%. In every case the desoxycholate salt was made up to the required concentration by dissolving it in the barbiturate buffer. One reading was made after one hour and another reading the next morning. The Beckman pH meter was used to check whether incorporation of the desoxycholate made
any appreciable change in the buffer, alcohol, neutral red system. No pH change was noted.

Another aspect of this experiment involved pretreating the organisms with the various concentrations of sodium desoxycholate for two hours and then removing the bile salt solution with two rinses of distilled water. The modified neutral red test was then performed on these pretreated cells. The pH of the aqueous sodium desoxycholate solution was adjusted to pH 7.

Results:

<table>
<thead>
<tr>
<th>Culture</th>
<th>Final Concentration of Sodium Desoxycholate</th>
<th>Time of Reading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25% 0.5% 1% 2% 0%</td>
<td></td>
</tr>
<tr>
<td>33605</td>
<td>white white white white white white white 1 hour</td>
<td></td>
</tr>
<tr>
<td></td>
<td>white white white white white white overnight</td>
<td></td>
</tr>
<tr>
<td>24.18</td>
<td>white white white white white white white 1 hour</td>
<td></td>
</tr>
<tr>
<td></td>
<td>white white white white white white overnight</td>
<td></td>
</tr>
<tr>
<td>H37Ra</td>
<td>white white white white white white white 1 hour</td>
<td></td>
</tr>
<tr>
<td></td>
<td>white white white white white white overnight</td>
<td></td>
</tr>
<tr>
<td>H37Rv and FD15</td>
<td>white white white white pink white 1 hour</td>
<td></td>
</tr>
<tr>
<td></td>
<td>white white white white white white pink red overnight</td>
<td></td>
</tr>
</tbody>
</table>

Two hour pretreatment with sodium desoxycholate pH 7 water (control) time of reading

<table>
<thead>
<tr>
<th>Culture</th>
<th>0.25% 0.5% 1% 2% 0%</th>
<th>pH 7 water (control)</th>
<th>Time of Reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>33605</td>
<td>white white white white white</td>
<td>1 hour</td>
<td>overnight</td>
</tr>
<tr>
<td></td>
<td>white white white white white</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.18 Avir</td>
<td>white white white white white</td>
<td>1 hour</td>
<td>overnight</td>
</tr>
<tr>
<td></td>
<td>white white white white white</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H37Ra</td>
<td>white white white white white</td>
<td>1 hour</td>
<td>overnight</td>
</tr>
<tr>
<td></td>
<td>white white white white white</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H37Rv</td>
<td>pink pink pink pink pink</td>
<td>1 hour</td>
<td>overnight</td>
</tr>
<tr>
<td></td>
<td>red red red red red</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Two hour pretreatment with sodium desoxycholate

<table>
<thead>
<tr>
<th>Culture</th>
<th>0.5%</th>
<th>1%</th>
<th>2%</th>
<th>0%</th>
<th>pH 7 water (control)</th>
<th>time of reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDL 5</td>
<td>pink</td>
<td>pink</td>
<td>pink</td>
<td>pink</td>
<td>pink</td>
<td>pink</td>
</tr>
</tbody>
</table>

Prewashing the cells with sodium desoxycholate solution has no effect. When sodium desoxycholate is incorporated in the modified system the otherwise positive cells react negative.

In the desoxycholate agar used in enteric work the organisms which produce acid from lactose precipitate the bile salt and show up as red opaque colonies. It was thought that if the H37Rv cells are more acid than the H37Ra cells the H37Rv cells should then precipitate the bile salt if it is incorporated into the neutral red test and in effect produce a more easily detectable red precipitate in the H37Rv cells. However, the results show that incorporation of sodium desoxycholate inhibited the virulent cells from showing any redness. However, when one pretreats the same cultures with sodium desoxycholate for 2 hours the virulent cultures react normally. It was thought, presuming there is some factor in the virulent cells that give them the property of giving a neutral red reaction, that the sodium desoxycholate had washed off this factor but the fact that pretreating the virulent cultures with sodium desoxycholate does not affect the neutral red reaction disproves this contention. Two possibilities remained: a) The bile salt has to be in conjunction with alcohol and the buffer to manifest its dissolving action. b) The bile salt does not dissolve
off the presumed neutral red factor but merely inhibits the absorption of the neutral red dye by the organisms. To test possibility (a) the supernatant mixture from the tubes with the H37Rv cells that had undergone the modified neutral red test which had sodium desoxycholate incorporated was discarded. The sediment cells were rinsed with distilled water and the modified neutral red test with no sodium desoxycholate was performed on these cells. All 5 tubes of the H37Rv showed a positive neutral red test. Thus possibility (a) was discounted. Possibility (b) was tested by discarding the supernatant mixture from the tubes with the FDL 5 cells that had undergone the modified sodium desoxycholate neutral red test, rinsing with distilled water and then adding 2 cc of 0.01 N HCl to the organisms. All of the clumps of organisms in the 5 tubes showed no red color so it was concluded that hypothesis (b) is valid. This inhibition of dye absorption was shown to be true with the avirulent cells also. This effect of sodium desoxycholate on dye absorption was shown to be true also with the standard neutral red test.

**Surface Active Properties of Sodium Desoxycholate**

Into a test tube was placed 1 cc of a 6% sodium desoxycholate-5%NaCl-1% sodium barbiturate solution, 1 cc of 0.1% neutral red solution and 1 cc of ether. Into a second test tube was placed 1 cc of 5% NaCl-1% sodium barbiturate solution, 1 cc of ether. In the first tube the dye took approximately 5 hours to penetrate the
ether layer. In the second tube where there was so sodium desoxycholate the dye penetrated into the ether layer within 2 minutes. Because of its surface active property, having both a water soluble and a fat soluble portion in its molecule, the sodium desoxycholate collects at the interface between the ether layer and the aqueous phase. This physical fact alone or the possibility of the neutral red being combined chemically with the sodium desoxycholate, hinders the neutral red from entering the ether phase. Neutral red is known to combine with sodium desoxycholate.

If we assume that the H37Rv or virulent cells have a neutral red factor which is some kind of an acidic constituent we can show in purely diagramatic form essentially what happens in the neutral red test. To do this 5 cc of 5%NaCl-1% sodium barbiturate and 1 cc of 0.01% neutral red was put into a tube and overlaid with 5 cc of ether. To a second tube 5 cc of 5%NaCl-1% sodium barbiturate-6% sodium desoxycholate, 1 cc of 0.01% neutral red was put into a tube and overlaid with 5 cc of ether. Into both tubes a strip of filter paper suspended by a string one third of its length into the ether layer. Both filter papers having been acidified with a few drops of acetic acid at the tip which is not submerged in the ether layer. We find that in the first tube the dye will have traveled into the ether layer up into the filter paper and turning red as it reached the acid within half an hour. In the second tube this does not happen even up to overnight. The filter paper in this setup represents the virulent tubercle bacilli, the
ether represents the fatty coating around the tubercle bacilli and the acetic acid represents the assumed neutral red factor.

**Use of Various Dyes at Different pH's in the Modified Neutral Red Test**

In place of the 1cc of 0.01% neutral red in the modified neutral red test, dyes with pH values not far from that of neutral red were used at pH'S slightly below and above their pH values. The results were read after one hour at room temperature and dye absorption was then determined by adding 3 cc of 0.1N HCl or 0.1N NaOH depending on whether the deeper color of the dye in question is on the acid or alkaline side of its pH value. H37Rv and H37Ra cells from 3 weeks old Loewenstein-Jensen slants were used.

In addition to this, with brom thymol blue, and brom-cresol purple, after the cells were treated with the respective dyes in buffer and alcohol for an hour they were subjected to various pH buffers to determine whether any differential color would result indicating a differential internal pH between the H37Rv and H37Ra cells.
Use of Cresol Red at Various pH's

Procedure:
clamp of respective Rv, Ra cells
1 cc of respective buffer
1 cc of 50% methanol
1 cc of .01% cresol red

<table>
<thead>
<tr>
<th>Test</th>
<th>Cells</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cresol red PK 6.8</td>
<td>Rv</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>yellow-red</td>
<td>Ra</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
</tbody>
</table>

Color of dye indicator
Buffer - alcohol mixture

<table>
<thead>
<tr>
<th>Discarding test supernatant and suspending cells in H2O as wash</th>
<th>Cells</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Ra</td>
<td>faint</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
</tbody>
</table>

Absorption test using 3 cc 0.1N NaOH
Note: Deeper color of indicator is at alkaline pH

<table>
<thead>
<tr>
<th>Absorption test using 3 cc 0.1N NaOH</th>
<th>Cells</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Ra</td>
<td>faint</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
</tbody>
</table>

Cresol red is not absorbed by H37Rv or H37Ra cells.
### Use of Chlor Phenol Red at Various pH's

<table>
<thead>
<tr>
<th>Test</th>
<th>Cells</th>
<th>PH 4</th>
<th>PH 5</th>
<th>PH 6</th>
<th>PH 7</th>
<th>PH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophenol red</td>
<td>Rv</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>PK 6 yellow-red</td>
<td>Ra</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Color of dye buffer -</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alcohol mixture</td>
<td>yellow</td>
<td>orange</td>
<td>red</td>
<td>purple</td>
<td>purple</td>
<td>purple</td>
</tr>
<tr>
<td>Discarding test supernatant and adding H₂O as a wash</td>
<td>Rv</td>
<td>yellowish</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>Ra</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Absorption test using 3 cc of 1N NaOH (Note: deeper color of dye on alkaline side of PK)</td>
<td>Rv</td>
<td>lavender</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>Ra</td>
<td>purple</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
</tbody>
</table>

At pH 4 chlor phenol red is absorbed to some degree by the H37Rv and H37Ra cells with apparently more dye absorbed by the latter.
### Using Phenol Red at Various pH's

**Procedure:**

1. Clump of Respective Rv and Ra cells
2. 1 cc of 50% methanol
3. 1 cc of buffer (respective)
4. 1 cc of phenol red

<table>
<thead>
<tr>
<th>Test</th>
<th>Cells</th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol red</td>
<td>PK 7.6</td>
<td>Rv</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>red</td>
<td>Ra</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>color of alc-buffer dye mixture</td>
<td></td>
<td>yellow</td>
<td>yellow</td>
<td>orange</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>Discarding test supernatant and adding H₂O as a wash</td>
<td>Rv</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>Ra</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Absorption test using 1N NaOH</td>
<td>Rv</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>Ra</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
</tbody>
</table>

Note: Deeper color of dye Ra is to alkaline PA.

Phenol red is not absorbed by H37Rv and H37Ra cells.
Use of Brom Cresol Purple at Various pH's

Procedure:
Clump of respective Rv, Ra cells
lcc of respective pH buffer
lcc of 50% methanol
lcc of .01% brom cresol purple

<table>
<thead>
<tr>
<th>Cells pH</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv white</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>white</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>white</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>white</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>white</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Brom cresol purple PK 6.3

<table>
<thead>
<tr>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv white</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>yellow</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Color of dye-buffer-alc mixture

<table>
<thead>
<tr>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>yellow</td>
<td>yellowish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>green</td>
<td>purple</td>
<td>purple</td>
<td>purple</td>
<td>purple</td>
</tr>
<tr>
<td>purple</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discarding test supernatant and suspending cells in H2O as wash

<table>
<thead>
<tr>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv yellow</td>
<td>white</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>greenish</td>
<td>greenish</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>yellow</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dye absorption test - by adding 3 cc .1N NaOH

<table>
<thead>
<tr>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv blue</td>
<td>light</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>blue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Deeper color of indicator dye is at alkaline PH.

<table>
<thead>
<tr>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra blue</td>
<td>dark</td>
<td>light</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>blue</td>
<td>bluish</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

At pH 5 and 6 there seems to be more brom cresol purple absorbed by the H37Ra cells than the H37Rv cells.
<table>
<thead>
<tr>
<th>Use of Brom Cresol Purple at pH 5-6</th>
<th>Cells</th>
<th>pH 5</th>
<th>pH 5.4</th>
<th>pH 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brom cresol purple PK 6.3</td>
<td>Rv</td>
<td>greenish</td>
<td>greenish</td>
<td>bluish</td>
</tr>
<tr>
<td>yellow-purple</td>
<td>Ra</td>
<td>yellow</td>
<td>yellow</td>
<td>yellow</td>
</tr>
<tr>
<td>0.01%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>color of dye-buffer alcohol mixture</td>
<td>Rv</td>
<td>yellow</td>
<td>greenish</td>
<td>purple</td>
</tr>
<tr>
<td>Discarding test solution</td>
<td></td>
<td>greenish</td>
<td>white</td>
<td></td>
</tr>
<tr>
<td>and suspending cells in water</td>
<td>Ra</td>
<td>yellow</td>
<td>greenish</td>
<td>grayish</td>
</tr>
<tr>
<td>Absorption test using</td>
<td></td>
<td>light</td>
<td>bluish</td>
<td>white</td>
</tr>
<tr>
<td>3 cc's of 0.1N NaOH</td>
<td>Rv</td>
<td>blue</td>
<td>blue</td>
<td>light</td>
</tr>
<tr>
<td></td>
<td>Ra</td>
<td>blue</td>
<td>blue</td>
<td>blue</td>
</tr>
<tr>
<td>brom cresol purple 0.1%</td>
<td>Test solution too intense in color; difficult to read color of cells.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>color of dye-buffer alcohol mixture</td>
<td>Rv</td>
<td>orange</td>
<td>red</td>
<td>purple</td>
</tr>
<tr>
<td>Discarding test solution</td>
<td></td>
<td>yellowish red</td>
<td>purple</td>
<td></td>
</tr>
<tr>
<td>and suspending cells in water</td>
<td>Ra</td>
<td>green</td>
<td>yellowish greenish</td>
<td>grayish</td>
</tr>
<tr>
<td>Absorption test using</td>
<td></td>
<td>blue</td>
<td>blue</td>
<td>light</td>
</tr>
<tr>
<td>3 cc of 0.1N NaOH</td>
<td>Rv</td>
<td>blue</td>
<td>blue</td>
<td>light</td>
</tr>
<tr>
<td></td>
<td>Ra</td>
<td>very-dark</td>
<td>dark</td>
<td>blue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>blue</td>
<td>blue</td>
<td>blue</td>
</tr>
</tbody>
</table>

There seems to be differential absorption of brom cresol purple by H37Rv and H37Ra cells at pH 5-6; at pH 6 and dye concentration of 0.01% dye is absorbed by H37Rv cells there is sufficient dye absorbed by H37Ra cells to give it a light blue color. Increasing the concentration of the dye the overall absorption of the dye by both the H37Rv and H37Ra cells but a differential absorption between the two is still manifested.
<table>
<thead>
<tr>
<th>Use of Brom Thymol Blue at Various pH's</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
</tr>
<tr>
<td>--------------------</td>
</tr>
<tr>
<td>Brom thymol blue PK 6.8 yellow blue</td>
</tr>
<tr>
<td>color of dye</td>
</tr>
<tr>
<td>buffer-alcohol mixture</td>
</tr>
<tr>
<td>Discarding test soln. and suspending cells in H2O</td>
</tr>
<tr>
<td>Absorption test using 3 cc of 0.1N NaOH</td>
</tr>
</tbody>
</table>

Using .01% brom thymol blue it is again seen that there is apparently more dye absorbed by the H37Ra cells than the H37Rv cells.

<table>
<thead>
<tr>
<th>Use of 0.1% Brom Thymol Blue at pH 8-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
</tr>
<tr>
<td>--------------------</td>
</tr>
<tr>
<td>Brom thymol blue 0.1% *</td>
</tr>
<tr>
<td>Color of dye-buffer-alcohol mixture</td>
</tr>
<tr>
<td>Discarding test solution and suspending cells in water</td>
</tr>
<tr>
<td>Absorption test using 3 cc of 0.1N NaOH</td>
</tr>
</tbody>
</table>

It was hoped that using a 0.1% solution of brom thymol blue and pH somewhere between pH 8-10 there would be such a great differenation in color between the H37Rv and H37Ra cells that the
latter would be white in color and the former a rather intense blue. If such was the case a new test to differentiate the two would be produced but the results did not warrant this.

* Difficult to read test due to intensity of color of test supernatant.

**Brom Cresol Purple**

<table>
<thead>
<tr>
<th>Test Solution</th>
<th>Cells</th>
<th>Test</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye 0.01% buffer</td>
<td>Rv</td>
<td>yellow</td>
<td>white</td>
<td>light</td>
<td>bluish</td>
<td>blue</td>
</tr>
<tr>
<td>pH 4 50% alc</td>
<td>Ra</td>
<td>yellow</td>
<td>light</td>
<td>bluish</td>
<td>blue</td>
<td>blue</td>
</tr>
</tbody>
</table>

Color of test solution yellow

<table>
<thead>
<tr>
<th>Test Solution</th>
<th>Cells</th>
<th>Test</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye 0.01% buffer</td>
<td>Rv</td>
<td>yellow</td>
<td>green</td>
<td>bluish</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>pH 5 50% methanol</td>
<td>Ra</td>
<td>yellow</td>
<td>light</td>
<td>bluish</td>
<td>light</td>
<td>light</td>
</tr>
</tbody>
</table>

Color of test solution greenish yellow

**Brom Thymol Blue**

<table>
<thead>
<tr>
<th>Test Solution</th>
<th>Cells</th>
<th>Test</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye 0.01% buffer</td>
<td>Rv</td>
<td>yellow</td>
<td>yellow</td>
<td>light</td>
<td>light</td>
<td>light</td>
</tr>
<tr>
<td>pH 4 50% methanol</td>
<td>Ra</td>
<td>yellow</td>
<td>blue</td>
<td>blue</td>
<td>blue</td>
<td>blue</td>
</tr>
</tbody>
</table>

Color of test solution yellow

<table>
<thead>
<tr>
<th>Test Solution</th>
<th>Cells</th>
<th>Test</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye 0.01% buffer</td>
<td>Rv</td>
<td>yellow</td>
<td>yellow</td>
<td>yellow</td>
<td>green</td>
<td>green</td>
</tr>
<tr>
<td>pH 6 50% methanol</td>
<td>Ra</td>
<td>yellow</td>
<td>green</td>
<td>yellow</td>
<td>blue</td>
<td>blue</td>
</tr>
</tbody>
</table>

Color of test solution yellow green

It was thought that this experiment might indicate whether there is a differential internal pH between the H37Rv and H37Ra
cells that is, the H37Ra cells have a higher internal pH accounting for the apparent greater coloration with the various dyes. The results show, however, that this is unlikely since if this was so increasing pH of the buffer treatment after its exposure to the dyes should make the H37Rv more similar in color with the H37Ra cells but this was not found to be the case.

**Incorporation of Neutral Red Into Loewenstein-Jensen Media**

H37Rv and H37Ra cells were inoculated respectively into 1 tube each of Loewenstein-Jensen medium containing 0.004% neutral red and into one tube each of Loewenstein-Jensen medium containing 0.002% neutral red.

H37Rv and H37Ra cells were inoculated respectively into 1 tube each of Loewenstein-Jensen media without malachite green but containing 0.002% neutral red final concentration.

All tubes were incubated at 37°C and at 1, 3 and 6 weeks all tubes were inspected for any differential colorization between colonies of H37Rv and H37Ra. There were no clear cut differentiation in color between the H37Rv and the H37Ra cultures. In all cases the colonies had a pink to red color depending on the concentration of neutral red in the medium.

**Modified Neutral Red Test Performed on Cells Pretreated With Brom Thymol Blue**

H37Rv cells and H37Ra cells from 3 weeks old Loewenstein-
Jensen cultures were treated with a mixture of 1 cc of 50% methanol, 1 cc of 0.1% brom thymol blue and 1 cc of pH 7 buffer for one hour. The cells were washed with water and then the modified neutral red test performed.

H37Rv and H37Ra cells from 3 weeks old Loewenstein-Jensen cultures were treated with a mixture of 1 cc of 50% methanol, 1 cc of 0.1% brom thymol blue and 1 cc of pH 9 buffer for one hour. The cells were washed with water and the modified neutral red test performed.

<table>
<thead>
<tr>
<th>pH 7</th>
<th>Mod. neutral red test</th>
<th>Absorption test</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>blue green</td>
<td>blue plus lavender pink</td>
</tr>
<tr>
<td>H37Ra</td>
<td>blue green</td>
<td>yellow green</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 9</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>blue</td>
<td>lavender pink</td>
</tr>
<tr>
<td>H37Ra</td>
<td>blue</td>
<td>yellowish green</td>
</tr>
</tbody>
</table>

Apparently H37Rv and H37Ra cells that have absorbed brom thymol blue are still capable of absorbing neutral red.

* In the absorption test with the H37Ra cells the cells turned red immediately after adding the 0.1N HCl, however, it took five minutes before these same cells would turn blue upon addition of 0.1N NaOH. Upon addition of acid again the cells would turn red immediately once more and upon addition of base the cells would again take five minutes before turning blue.

Incorporation of Brom Thymol Blue and Neutral Red in Loewenstein-Jensen Media

H37Rv and H37Ra cells are inoculated respectively into 2
tubes each of Loewenstein-Jensen medium with no malachite green but containing 0.04% brom thymol blue and 0.04% neutral red; Loewenstein-Jensen media with no malachite green but containing 0.08% brom thymol blue and 0.04% neutral red; Loewenstein-Jensen media with no malachite green but containing 0.04% brom thymol blue and 0.08% neutral red; Loewenstein-Jensen media containing no malachite green but 0.02% each of brom thymol blue and neutral red. These were incubated at 37°C and at one, three and six weeks they were examined. Differential colorization between the H37Rv and H37Ra colonies were not noted. All colonies took up the red color of neutral red. However, it was thought that there might be a differential decolorization with alkali. Thus H37Rv colonies and H37Ra colonies taken from the culture slants containing 0.02% each of brom thymol blue and neutral red were treated with 0.1N NaOH. Both the H37Rv and H37Ra colonies became instantly white; when treated with the barbiturate buffer of the neutral red test both the H37Rv and H37Ra colonies became white in a few minutes.
CHAPTER V

GENERAL DISCUSSION

A study of the neutral red test has been made with the purpose of contributing to an understanding of the mechanism of this test and also to introduce a simpler, modified neutral red test. The problem is thus twofold and though related, for the sake of organization, an attempt has been made to keep them separate.

In the beginning it was thought that there were three possible explanations for the neutral red cytochemical test. They were thought to be: a) The virulent cells possesses some kind of a metabolic process, governed by an enzyme which reduces the neutral red, whereas the avirulent cells lack this metabolic process or enzyme. b) The virulent cells possess some chemical constituent which is acidic in nature and is lacking or in lesser amount in the avirulent cells. c) There is a difference in permeability between the virulent cells and the avirulent cells.

With these three possible explanations in mind it was hoped that by studying the conditions and constituents necessary in performing the neutral red test some understanding of the mechanisms of the test would result. It was also thought that some insight into the mechanism of the neutral red test would be ob-
tained by determining what experimental procedures would destroy the ability of the virulent cells to give a positive neutral red test.

The first question considered was the significance of the 50% methanol pretreatment in the neutral red test. Whether this pretreatment was eliminated, or substituted with distilled water pretreatment, both the H37Rv and H37Ra cells showed a positive reaction (pink color). However this color was not as deep as the red color of the H37Rv cells when the 50% methanol pretreatment was used. It was later noticed that when the test was read after 3 hours instead of 1 hour the original pink color of the H37Ra cells would disappear. It seems that the tween 80 albumin medium itself is on the acid side of neutral red since upon adding a few cc's of the medium to a mixture of the barbiturate buffer and neutral red a reddish tinge would be seen in the originally amber colored solution. It was also noted that upon addition of a few ml of tween albumin medium (whether fresh, uninoculated media or media in which H37Rv or H37Ra cells have been growing) to H37Ra cells that had given a negative neutral red test a pink positive color would result. Absorption of the neutral red does not seem to be as great in cells not pretreated with the 50% methanol. This was determined by adding a few ml of 0.1 N HCl to cells that had undergone the neutral red test.

The 50% methanol pretreatment removes what media may be clinging to the cells which might give the H37Ra cells a false
positive reaction and it also in some manner makes the H37Rv cells absorb more dye and subsequently a stronger positive neutral red reaction. The concentration of methanol can be varied from 30 to 95% and yet have good differentiation between the H37Rv and H37Ra cells. Other chemicals such as isoamyl alcohol, isopropyl alcohol, butyl alcohol, pentane or petroleum ether could be used in place of the 50% methanol.

The concentration of neutral red and barbiturate buffer as used in the neutral red test seems to be optimal. Decreasing the concentration of the neutral red decreases the color of the H37Rv cells and increasing the concentration gives the H37Ra cells a pink color. Decreasing the buffer concentration gives the avirulent cells a pink color and increasing the buffer concentration lessens the color of the H37Rv cells.

The next problem studied was the pH of the neutral red test. It was found that at pH 9.0 and below both the H37Rv and H37Ra cells are colored red. It is possible that at pH 9.0 and below there is present sufficient concentration of the red undissociated form of neutral red which merely absorbs onto the H37Ra cells giving them a red color. However, it is also possible that the H37Ra cells are not totally deficient in that factor which is responsible for the H37Rv cells giving a positive neutral red reaction. Thus with the H37Ra cells the factor may be manifested only when the pH of the system is brought down to 9.0 or below.
In any case the possibility that neutral red is not absorbed by the H37Ra cells can be dispensed with since a red color is manifested after adding 0.1N HCl to H37Ra cells that had previously undergone the neutral red test. It was thought, however, that the H37Ra cells may not absorb as much dye as the H37Ra cells but contrary to what was expected it was shown that there is more dye absorbed by the H37Ra cells.

Dubos and Middlebrook, 1948, specifies room temperature for the performance of the neutral red test. It has shown, however, that 25°C, 30°C or 37°C can be used with equally good differentiation between the H37Rv and the H37Ra cells. The color of H37Rv cells however seems to be more intense when the test is performed at 37°C. The intensity of the red color at room temperature is sufficient and for convenience sake it would be just as well to perform the test at room temperature. However, in the modified neutral red test 30°C or 37°C shows a definite improvement in the intensity of color in the H37Rv cells over that when performed at room temperature. Since in the diagnostic laboratory you would likely find a 37°C incubator it would be better to use 37°C rather than 30°C as the latter may not be in use in most laboratories. Incubator temperature has the advantage of being constant whereas the room temperature is not constant. The effect of increasing the temperature on the intensity of the color of the H37Rv cells is more apparent in the modified neutral red test because here the color varies from a pink to a pink-red color whereas
in the standard neutral red test the color varies from lavender red to red which is not as noticeable. At 8°C the color of H37Rv cells is pinkish at 1 hour and, whereas when performed at higher temperatures there is a pinkish color as early as 10 minutes the H37Rv cells is white even up to 30 minutes. This gives the impression that at 8°C the reaction is slowed down. It is interesting to note that when the H37Ra that have undergone the neutral red test at 8°C were taken out from its 1 hour stay in the icebox and put into the 56°C water bath for 10 minutes the pinkish color that it had when in the icebox disappears. Following the same procedure with the H37Rv cells the pinkish color that was present after 1 hour at 8°C was intensified to a pink color after 10 minutes at 56°C. Again suggesting that the reaction is slowed down at low temperatures and speeded up at higher temperatures. At 56°C however the reaction seems to be inhibited in that the H37Rv cells no longer show any trace of red.

Apparently chemically or heat killed H37Rv and H37Ra cells can be differentiated just as well as unheated or non-chemically treated H37Rv and H37Ra cells. It seems unlikely that the neutral red reaction is due to any living process within the cells or dependent upon enzymes. Placing a water suspension of H37Rv cells in a boiling water bath for 1 to 2 hours either destroys or extracts that factor which makes H37Rv cells color red in the neutral red test. This effect of heat is not evident when the H37Rv cells are heated dry for 2 hours.
Recently methods have been developed whereby proteolytic enzymes have been used in the isolation procedure of *Mycobacterium tuberculosis* and this was the reason for studying if proteolytic enzyme treated H37Rv and H37Ra are still able to be differentiated with the neutral red test. Whether treated with pepsin, trypsin or papain the H37Ra and H37Rv cells still gave clear-cut differential color reactions with the neutral red test.

The neutral red which is absorbed by the H37Rv and H37Ra is capable of being extracted with methanol and various agents. The extracting ability of the methanol being greater as the concentration of alcohol is increased. The dye which is absorbed by the H37Rv cells seem to be extractable just as readily as the dye which is absorbed by the H37Ra cells. The extraction of the dye apparently does not disrupt the H37Rv cells in anyway with respect to their ability to give a positive neutral red test. They will again give a neutral red positive test if a second neutral red test is performed after the dye from the first test has been extracted. If there is a specific chemical constituent in the H37Rv cells (which is found to be so later on) which is responsible for a positive neutral red test the combination of the dye and this constituent is apparently reversible.

While studying whether the neutral red is absorbed by H37Ra cells it was first noticed that there is a differential time of decolorization with 0.1N NaOH when applied to H37Rv and H37Ra cells that had undergone the neutral red test, the H37Ra cells
being acidified after the neutral red test to manifest the dye that is absorbed. Two theories could be postulated to explain this: The dye which is absorbed by the H37Ra cells is not chemically bound whereas the dye which is absorbed by the H37Rv cells is bound chemically to some constituent in the cells. Thus decolorization in the H37Ra is merely dependent on entrance of the alkali into the cells to decolorize what unbound dye may be there. However, in the case of the H37Rv cells the alkali not only has to enter the cells but must also replace or break the bond between the absorbed dye and the chemical constituent to which it is bound. The second possibility which could be postulated is that the H37Ra cells are more permeable to the alkali than the H37Rv cells since it has already been shown that there is a difference in permeability between the H37Ra and the H37Rv cells. There is significantly more neutral red absorbed by the H37Ra cells. In this respect it is interesting to note that in 1956 D'Arcy and Rees explained that the methylene blue virulence test was not so much the lack of dehydrogenase enzyme in the virulent organisms but rather due to a difference in permeability between the virulent and avirulent organisms. By treating the virulent organisms with surface active agents in concentrations which might be expected to increase the permeability of the cell they found that they could reduce the decolorization time of methylene blue by the virulent organisms from 90 minutes to 29 minutes which is approximately the time required for avirulent cultures to decolorize the methylene blue.
If there is a greater permeability in the H37Ra cells as compared to the H37Rv cells it is conceivable that it might play an important part in the neutral red test. In support of this idea the present author has found that sonic treated H37Rv cells will give a negative neutral red test as the H37Ra cells in spite of the fact that a substance that will give a positive neutral red test can still be extracted from them. It seems the explanation lies in the fact that though the cells still possess the lipid substance which will give a positive neutral red test, their permeability has been altered by the sonic vibration. In support of this is the fact that H37Rv cells that have given a positive neutral red test and subsequently sonic treated will be decolorized immediately upon introduction in 0.1N NaOH or the barbiturate buffer. This is true also of H37Ra cells that have undergone the neutral red test and then acidified to manifest a red color. In contrast H37Rv cells colored red by the neutral red test but not sonic treated will not be decolorized by 0.1N NaOH or the barbiturate buffer until 1 hour or longer.

Knaysi (1941) indicated that neutral red could be used to distinguish between fats and fatty acids. The principle being that fats are stained yellow by more absorption of the dye base whereas fatty acids are stained red by forming a salt with the neutral red. Thus it is conceivable that the basis of the neutral red virulence test follows along this same principle, that some fatty acid constituent in the virulent cells is responsible for
their giving a red color in the neutral red test. Somewhat supporting this theory is the observation that hydrolysis with HCl, which could conceivably hydrolyse this hypothetical fatty acid, will make the H37Rv cells lose their ability to give a positive neutral red test.

Using ether, a lipid like substance has been extracted from H37Rv cells which substance seems to be lacking in the H37Ra cells. The substance was not soluble in methanol or water but soluble in ether and chloroform. This substance seems to be responsible in some way for the H37Rv cells giving a positive neutral red test as the lipid like substance itself will give a positive neutral red test. When extracted H37Rv cells, or unextracted or extracted H37Ra cells were coated with this substance they give a positive neutral red test where formally they were neutral red negative.

A modified neutral red test has been proposed in which the alcohol pretreatment is eliminated by incorporating the alcohol with the neutral red and buffer in a 1:1:1 proportion. A comparative study of this modified test and the standard neutral red test has shown close correlation between the two. It is possible that the modified method is not as sensitive as the standard method in that it will not detect those cultures which have very little of the lipid like substance found in the virulent cells. This however does not detract from its usefulness as a preliminary screening test. There are indications that by using 1 cc of 30% methanol
instead of 50% methanol in the modified test and performing the test at 37°C instead of at room temperature the test could be increased in sensitivity. In the author's opinion this would improve the modified neutral red test. The possibility exists also that by increasing the pH of the buffer the methanol can be omitted entirely.

An unsuccessful attempt has been made to incorporate the neutral red in the Loewenstein-Jensen medium to produce a neutral red test in the culture as it grows out.

Also unsuccessful was the attempt to use various other dyes in place of the neutral red. However these experiments did point out that the H37Ra cells seem to be more permeable than the H37Rv cells since it was shown that the H37Ra cells absorb more brom purple or brom thymol blue than the H37Rv cells.

It has been shown also that brom thymol blue (an acid dye) and neutral red (a basic dye) can be located in the same cells at the same time. It is possible that the substance in the cells which absorb the basic dye neutral red is superficial to the substance which absorbs the acid dye as the neutral red is manifested immediately upon adding acid to cells that were treated with neutral red and brom thymol blue whereas these same cells will manifest the brom thymol blue only after 5 minutes after adding the NaOH.
CHAPTER VI

SUMMARY AND CONCLUSION

By varying the experimental conditions under which the Dubos-Middlebrook neutral red test is performed and by pretreating the H37Rv and H37Ra cells by various physical and chemical means prior to having the neutral red test performed on them several interesting facts regarding the mechanism of the test and the conditions under which the test should be performed were realized.

A lipid-like substance that will give a positive neutral red test can be extracted from the H37Rv cells.

It has been shown that H37Rv cells treated with sonic vibration will give a negative neutral red test in spite of the fact that a substance that will give a positive neutral red test can still be extracted from them. The extraction of this substance from the sonic treated H37Rv cells and from untreated H37Rv cells was accomplished with the use of diethyl ether for 24 hours at 37°C. On the other hand little or no detectable amount of this substance can be extracted from the H37Ra cells.

Interestingly enough, H37Ra cells that have undergone the neutral red test and treated with a weak acid to manifest the neutral red dye that it has absorbed will be decolorized by NaOH within several minutes whereas H37Rv cells under the same condition will take a hour or longer to be decolorized. Likewise, H37Rv
cells that give a positive neutral red test and subsequently sonic treated will behave identically as H37Ra cells in being rapidly decolorized when immersed in 0.1N NaOH or the 1% barbiturate buffer.

The theory is proposed that sonic treatment of the H37Rv cells increase the permeability of these cells such that their permeability approach that of the H37Ra cells and are thus decolorized as readily as the H37Ra cells. It is believed that this difference in permeability between the H37Rv and H37Ra cells play an important role in the neutral red test.

In conclusion it can be said that there are two factors which seem to be responsible for the neutral red test. One, is the lipid-like substance which is extracted with ether from the H37Rv cells. Two, is the difference in permeability between the H37Rv and H37Ra cells. The H37Ra cells are more permeable than the H37Rv cells thus facilitating entrance of the buffer into the H37Ra cells.

By incorporating the 50% methanol along with the buffer and neutral red in a 1:1:1 proportion and eliminating the alcohol prewashing a modification of the neutral red test has been proposed which by comparative study correlates well with the standard neutral red test and could very well be applied as a screening virulence test.


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APPROVAL SHEET

The dissertation submitted by Harry Youmun Wong has been read and approved by a committee of five members of the Graduate Faculty.

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.

January 15, 1958

Date

Signature of Advisor