A CRITICAL SURVEY AND EXPERIMENTAL STUDY OF METHODS OF ISOLATING TUBERCLE BACILLI FROM SPUTUM

THESIS FOR THE DEGREE OF DOCTOR OF MEDICINE PRESENTED BY

A. J. O'HEA, M.B., Ch.B.

VOLUME I: Text

PREFACE

This thesis is presented in 2 volumes: volume I contains the text and volume II the tables and illustrations. In addition to the general table of contents at the beginning of volume I, each of the longer chapters of the text carries its own particular table of contents. In volume II, tables 1 to 54 summarise the results of authors whose work is discussed in the review of the literature; tables 55 to 88 summarise my own work.

Experiments with the tubercle bacillus are notorious for their time-consuming nature and for the frequency with which they yield disheartening results. My labours, both mental and physical, proved to be no less than I had been warned to expect, but I must emphasise that many of the experiments would not have been possible without some co-operation from others. It is with great pleasure that I remember and acknowledge my sources of assistance and encouragement.

This work was undertaken primarily because Professor J. W. Howie drew my attention to the urgency of laboratory problems concerned with the diagnosis, treatment and control of tuberculosis; the work was done while I was a member of his staff. His advice and encouragement lightened the periods of depression which are inevitable in the type of investigations required for this thesis; the equipment and technical assistance available in his department did much to ease the physical part of my labours.

For the purposes of my investigations it was essential to have access to patients suffering from pulmonary tuberculosis. This important part of my work was facilitated by the courtesy and understanding of Dr. A. W. Lees and the staff of the tuberculosis division at Ruchill Hospital, Glasgow. I must also record my gratitude to the patients under the care of Dr. Lees for their unfailing co-operation in the provision of specimens.

Discussion with other workers helped me to determine my approach to certain problems. In particular I am indebted to the following: Dr. E. Nassau, Harefield Sanatorium, Middlesex, who drew my attention to the possible value of his swab method which I investigated in experiment 12; Dr. D. D. Reid, London School of Hygiene and Tropical Medicine, for access to unpublished information concerning the incidence of pulmonary tuberculosis in laboratory workers; and Dr. W. McNaught, Ruchill Hospital Laboratory, Glasgow, for access to unpublished information on a simple method of determining the sensitivity of tubercle bacilli to anti-tuberculous drugs.

I wish to thank the donors of certain materials used in my experiments: Dr. N. R. Grist, Department of Virology, Ruchill Hospital, Glasgow, for a supply of Mycostatin; Burroughs Wellcome & Co. Ltd. for a supply of Polymyxin B sulphate; Sharp and Dohme, Ltd., for a supply of Bacitracin; and Geigy, Ltd., for a supply of Desogen.

The illustrations for the thesis were prepared in the Departments of Pathology and Bacteriology of Glasgow University. The photographs were taken by Mr. G. Kerr and the coloured drawings were made by Mr. R. Callander; I am indebted to both for their skill and co-operation.

I am indebted to the Rankin Research Fund for a grant towards the expenses of this work.

I wish to thank Miss E. McGlone for her patience, co-operation, and courtesy in the preparation of this thesis for publication.

Robroyston Hospital Laboratory,
September, 1957.

A. J. O'Hea

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INTRODUCTION

The examination of sputum for the presence of tubercle bacilli is an important part of the work done by hospital laboratories. Before the introduction of the chemotherapeutic agents which have profoundly changed the results of treatment of tuberculosis, the aim of laboratory investigations was chiefly diagnostic: to establish or confirm a diagnosis of pulmonary tuberculosis; to assess the activity of a pulmonary lesion thought to be tuberculous; and to determine whether or not a tuberculous patient was a dangerous source of infection to others. Although the most rapid method of demonstrating tubercle bacilli is the microscopic examination of a suitably stained smear of sputum, the most sensitive method is the isolation of these organisms by culture or by the inoculation of a guinea pig.

The introduction of para-aminosalicylic acid, streptomycin and isoniazid for the treatment of tuberculosis has increased the importance of isolating tubercle bacilli. The use of these drugs is complicated by the emergence during treatment of tubercle bacilli resistant to the agent or agents employed. It has been established by the Tuberculosis Chemotherapy Trials Committee of the Medical Research Council (1953b) that, during treatment, the incidence and emergence of strains of tubercle bacilli resistant to chemotherapeutic agents is greatly reduced by using these agents in combination rather than singly. The combination of two agents

does not prevent the development of resistance if the patient's strain is initially resistant to one of the agents. Therefore, before treatment begins, it is highly desirable to isolate tubercle bacilli from the patient in order that he may be treated with a suitable combination of anti-tuberculous drugs determined by testing the sensitivity of his organisms to these drugs. The isolation of tubercle bacilli is also important in assessing the response of a patient to chemotherapy. One of the most favourable prognostic signs is the rapid elimination of tubercle bacilli from the sputum. During treatment - especially if the patient makes disappointing progress - it may be necessary to isolate tubercle bacilli for tests of sensitivity to anti-tuberculous drugs in order to establish whether the patient's organisms have become resistant to the drugs employed and, if this is the case, to indicate the appropriate agents for further chemotherapy.

The common methods of isolating tubercle bacilli from sputum involve shaking of the sputum with an agent which kills organisms other than tubercle bacilli. Usually sodium hydroxide or sulphuric acid is used for this purpose. These decontaminating procedures also homogenise the specimen and permit concentration of the tubercle bacilli by centrifugation. Such methods are far from ideal: (1) they are time-consuming and demand a high standard of technical skill on the part of the laboratory worker; (2) they are dangerous because a highly

infective aerosol is likely to be released when a specimen containing many viable tubercle bacilli is homogenised by shaking with a decontaminating agent; (3) although decontaminating agents are used to provide an inoculum free from live organisms other than tubercle bacilli, they also reduce the number of viable tubercle bacilli in the inoculum. These undesirable features of the common decontaminating procedures assume much greater importance when a laboratory has to deal with large numbers of specimens. The advent of specific chemotherapy for pulmonary tuberculosis has recently increased the demand for laboratory examinations of sputum to such an extent that, in this country, laboratories serving tuberculosis clinics and sanatoria have to cope with numbers of specimens greatly in excess of the number which can be handled safely and efficiently by present methods. This problem is even more acute in countries which do not have well-developed laboratory services. The tendency in some overworked laboratories to operate these methods with young members of technical staff who have not completed their training is a source of great danger to these trainees and to others working in their vicinity.

It is important, therefore, to re-examine methods of isolating tubercle bacilli taking into account not only the factor of efficiency, but also the factors of simplicity and safety. This thesis presents: (1) a critical review of bacteriological methods for the control of tuberculosis and its chemotherapy with special reference to factors which

make for safety in large-scale work in laboratories which are not specially designed to prevent the exposure of technical staff to aerosols likely to contain numerous viable tubercle bacilli; (2) methods of assessing the efficiency of decontamination procedures for the isolation of tubercle bacilli from sputum; and (3) a critical examination of the efficiency of a number of methods of isolating tubercle bacilli from sputum.

LITERATURE

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SCOPE OF LITERATURE REVIEWED

The most important pre-requisite to the isolation of tubercle bacilli from patients suffering from pulmonary tuberculosis is to ensure that the inoculum employed has been freed from organisms other than tubercle bacilli - in other words, the sputum must first be efficiently decontaminated. Other factors - for example, the choice of medium, the number of tubes of medium inoculated, the inclusion or omission of guinea pig inoculation - have an important influence on the efficiency with which tubercle bacilli will be isolated from the specimen. These additional problems will not, however, be treated in detail for two reasons. First, the method adopted for the decontamination of the specimens of sputum determines 1) the time spent in the routine isolation of tubercle bacilli from sputum and 2) the safety of the procedure. Second, the lethal effect on tubercle bacilli of the decontamination procedure exerts an important influence on the results obtained with artificial media, with animal inoculation, or with a combination of these two procedures. This review of the literature will therefore be limited to the following topics: (1) the efficiency of standard methods of isolating tubercle bacilli from sputum; (2) new methods of isolating tubercle bacilli from sputum;

(3) the place of micro-culture methods in routine laboratory practice; (4) methods adopted when sputum is not available for the isolation of tubercle bacilli from patients suffering from pulmonary tuberculosis; and (5) the safety and simplicity of these and other laboratory procedures adopted for the diagnosis, treatment, and control of pulmonary tuberculosis.

For the convenience of the reader, I have tabulated the most important results of trials of methods of isolating tubercle bacilli from sputum. In my tables, the presentation of results often differs from that given in the original papers. It was necessary to compile these tables for the following reasons. First, I have adopted a fairly uniform scheme of presentation in order to facilitate comparison of the results of different studies. Second, the results reported in some of the studies in this review were either not tabulated or poorly tabulated and therefore required clarification. Third, it is important to know the type of specimens with which a given result was obtained because specimens containing few tubercle bacilli show differences between methods of isolating tubercle bacilli more clearly than specimens containing many tubercle bacilli; the tables which I have compiled either indicate the type of specimen used or indicate that information concerning the type of specimen has not been given in the original paper.

EFFICIENCY OF STANDARD METHODS OF ISOLATING TUBERCLE BACILLI FROM SPUTUM

The development of methods of isolating tubercle bacilli from sputum.

Antiformin, a mixture of equal volumes of 15 per cent. sodium hydroxide solution and liquor sodae chlorinatae (B.P.) was the first agent used for the isolation of tubercle bacilli from infected material (Uhlenhuth and Xylander, 1909); Griffith (1914) introduced the method to this country. Because the antiformin method failed "to give a rapid and uniform growth" and because "repeated washing of the sputum with sterile water diminishes the number of organisms and increases the possibility of contamination," Petroff (1915) introduced the use of sodium hydroxide. He treated the sputum with an equal volume of 3 per cent. sodium hydroxide for from twenty to thirty minutes, neutralised the mixture with normal hydrochloric acid and inoculated media with the deposit obtained by centrifuging. In his hands the method yielded 69 cultures from 69 specimens of sputum, 6 of which were negative for acid-fast bacilli by microscopy. Löwenstein (1924) suggested the use of sulphuric acid. His results - like those of Griffith with antiformin - indicate only that the method is feasible and do not compare this method with any other method. Corper and Uyei (1927) preferred treatment with 6 per cent. sulphuric acid to treatment with 2 per cent. sodium hydroxide and later, (1930), suggested the use of 5 per cent. oxalic acid instead of mineral acid. They claimed that oxalic acid had

3 advantages: (1) it was available as a chemically pure solid from which solutions of the required strength could be made up rapidly and accurately; (2) it was less toxic to tubercle bacilli than sulphuric acid; (3) it controlled contamination more effectively than sulphuric acid. In 1938 Jungmann and Gruschka described an acid-iron-peroxide method for the homogenisation of sputum for microscopy and culture. This method gave satisfactory results in the hands of Nassau (1942), and became popular in this country. To 5 ml. of sputum are added 3 ml. of a solution of 10 per cent. ferrous sulphate in 10 per cent. (V/V) sulphuric acid and 3 ml. of a 3 per cent. solution of hydrogen peroxide. The exact concentration of hydrogen peroxide in the original method is considered by Anderson et al. (1953) to be equivalent to a 10 volume solution hydrogen peroxide; they emphasise that Nassau specified a concentration of 1 volume per cent. which they presume means a 1 volume strength solution and that this weaker concentration is the one which has been erroneously adopted by most workers in this country. Corper and Stoner (1946) suggested the use of trisodium phosphate because they found it less toxic to tubercle bacilli than sodium hydroxide or oxalic acid; they recommend treatment of the specimen with trisodium phosphate for 24 hours at 37°C.

Although the antiformin method is still used for the cultivation of tubercle bacilli in some laboratories, most workers have discarded it in favour of one of the other methods. Griffith (1916) drew attention to the fact that

in "thin" sputa "tubercle bacilli themselves did not long resist the action of 5 per cent. antiformin. In 13 experiments mixtures which yielded pure cultures of tubercle bacilli after 10 minutes' exposure to 5 per cent. antiformin were sterile after further periods of 5 or 10 minutes." Lurie (1923) showed that the antiformin method was inferior to Petroff's sodium hydroxide method (table 1) and Saenz and Paterson (1938) found a final concentration of 10 per cent. antiformin inferior to sulphuric acid used in 7.5 per cent. final concentration. Soltys (1952) classified it under "methods to be used only before direct microscopy."

The other methods have been investigated by numerous workers but these studies have produced no general agreement; the results obtained by one worker often contradict those of another. There are many possible reasons for this state of affairs. Two different general methods of trial have been adopted: (1) a comparison of two or more methods using large number of specimens of sputum and (2) quantitative studies of the lethal effects on tubercle bacilli of two or more methods.

The first method seems ideal - "the proof o' the puddin's the preein' o't" - but the following disadvantages must be borne in mind. First, the numbers of specimens must be large enough to yield statistically significant results; second, if specimens containing large numbers of tubercle bacilli are used, the differences between methods may be minimised and if a series of specimens containing small numbers

of tubercle bacilli is used it will probably contain a large number of specimens incapable of yielding positive results by any method; and third, this kind of trial only relates one method to another and does not establish the efficiency of any method in absolute terms. The second method can be made to yield absolute results concerning the lethal effect of the agents under trial but so far these values have been established only with aqueous suspensions of tubercle bacilli or with non-tuberculous sputum to which has been added a known number of tubercle bacilli; it is doubtful if such studies provide valid results for the assessment of methods to be used in routine laboratory practice.

Apart from the difficulties introduced by having two main ways of assessing decontamination methods, it is often impossible to compare the results of one worker with those of another for the following reasons: (1) because the details of these methods are easily modified, (2) because different media are employed by different workers and (3) because the results are often recorded with insufficient detail (see p. 8 of this thesis). It is proposed therefore to present the results obtained with specimens of sputum in a recent carefully controlled trial conducted by the Public Health Laboratory Service (1952) and, in the light of the results of this trial, to examine (1) the results obtained with these methods by other workers who used series of sputum specimens; and (2) the results obtained in quantitative assessments of the lethal effects on tubercle

bacilli of these decontaminating agents.

Evaluation of Standard Methods

Public Health Laboratory Service Trial (1952). The methods examined in this trial were (1) treatment of the sputum with an equal volume of 3 per cent. (V/V) sulphuric acid at 37°C for not more than 30 minutes, (2) treatment with an equal volume of 4 per cent. sodium hydroxide until homogenisation was complete, (3) treatment with acid-iron-peroxide, using a concentration of hydrogen peroxide of 1 volume strength (see p. 10 of this thesis) for 20 minutes at room temperature and (4) treatment with trisodium phosphate for 24 hours at 37°C. Twelve laboratories took part in the trial but all controllable details of procedure were carefully standardised throughout the 12 laboratories. In each laboratory the methods were to be tested in pairs on groups of 52 specimens and the 6 possible combinations of pairs from the 4 methods were tested in each laboratory; a few laboratories were unable to complete this programme for every pair of methods under trial. The trial was planned in 6 stages, arranged so that in any stage all the possible combinations were tested, each combination being assigned to 2 of the 12 laboratories. The specimens of sputum examined at each stage by each laboratory were selected so that roughly three quarters were negative by microscopy for acid-fast bacilli and the remainder were positive by microscopy. The microscopically

negative specimens were selected from out-patient tuberculosis clinics and sanatoria; the microscopically positive specimens were defined as those "in which, on careful examination of 100 fields, 20 or less than 20 organisms or clumps of organisms morphologically resembling Mycobacterium tuberculosis were detected." Each specimen in the trial was therefore examined by 2 methods, the specimen being divided equally between the two methods. The number of examinations reported in the trial is therefore twice the number of specimens used. In addition to the main results of the trial (table 2), it was shown that the division of the sputum into 2 portions did not bias the results; first and second portions of specimens yielded comparable numbers of positive results.

The trial placed the four methods in the following order of efficiency: (1) the sodium hydroxide method; (2) the sulphuric acid method; (3) the acid-iron-peroxide method and (4) the trisodium phosphate method (table 2). The order is the same for specimens negative by microscopy, specimens positive by microscopy and for all specimens taken together. Significant differences between the methods, however, are only obtained with microscopically negative specimens: the sodium hydroxide method is significantly superior to the three other methods and the sulphuric acid method is significantly superior to the trisodium phosphate method.

In the introduction of the paper reporting this investigation, attention is drawn to the contradictory nature of the literature on methods of preparing sputum for the inoculation of media; the discussion of the results,

however, makes no attempt to examine the results of other workers and it is simply stated that "the conclusions are valid only for the technique as described including the use of Löwenstein-Jensen medium" and that "with variations in the technique or the use of different media different results might have been obtained, affecting the order of merit assigned to the various methods." This cautious attitude is justified to some extent because it is difficult to compare results obtained by different kinds of trials carried out under conditions which may differ greatly from one laboratory to another. Nevertheless, failure to examine the results obtained by other workers suggests (1) that the significant difference between the sodium hydroxide method and the other methods is some kind of artefact depending entirely on the details of procedure adopted for the other methods and (2) that modification of one of the other methods might make it as efficient or significantly more efficient than the sodium hydroxide method.

Other trials with series of sputum specimens. The following methods were tested by Baker (1951): (1) treatment with sodium hydroxide combined with flocculation (Hanks et al. (1938), (2) treatment with 5 per cent. oxalic acid, (3) treatment with trisodium phosphate, (4) treatment with acid-iron-peroxide, using 1 volume strength hydrogen peroxide and (5) treatment with sodium hydroxide. Using a table of random numbers, specimens of sputum were allocated to each method in batches of 12; each specimen was cultured only by

one method. The results in table 3 are taken from Baker's paper. The number of specimens tested by each method is small. In addition, comparison of the results of these small series is made less profitable by the fact that the specimens used in each series were different. Such as they are, the results agree with those obtained by the Public Health Laboratory Service in placing the sodium hydroxide method first in order of efficiency, especially for specimens negative for acid-fast bacilli on direct microscopy; on the other hand, the oxalic acid method used by Baker is inferior to the trisodium phosphate method whereas the sulphuric acid method used by the Public Health Laboratory Service was significantly superior to the trisodium phosphate method for specimens negative by direct microscopy. In this connection it must be noted that the exposure time adopted by Baker was 40 minutes at 37°C for oxalic acid and that the corresponding time for sulphuric acid in the Public Health Laboratory Service trial was never more than 30 minutes at 37°C. There seems to be little advantage in the modification of the sodium hydroxide method proposed by Hanks et al. and the results obtained with the acid-iron-peroxide method are very poor for specimens negative on direct microscopy.

Most of the other authors who use series of specimens in trials of methods of preparing sputum for culture limit the study to a pair of methods. These trials will be considered under the following headings:-

a) Comparisons between alkali methods.

b) Comparisons between acid methods.

c) Comparisons between alkali methods and acid methods.

a) Comparisons between alkali methods.

At first sight it seems strange that the sodium hydroxide method should be so much better than another alkali method, the trisodium phosphate method, in the hands of the Public Health Laboratory Service (table 2). This result confirms that of Mullahy (1950, table 4) who found a difference between the two methods which agrees closely with that found by the Public Health Laboratory Service. A striking superiority of the sodium hydroxide method was also demonstrated by Byham (1950, table 5). Mullahy and Byham, however, experienced very high contamination rates with the trisodium phosphate method (30.4 per cent. and 20.2 per cent. respectively); these contamination rates bias their results in favour of the sodium hydroxide method.

On the other hand Starkey and Aubert (1950) claim better results with trisodium phosphate than with sodium hydroxide for specimens negative by microscopy. Their results (table 6) do not show a great difference between the two methods except when trisodium phosphate is used as a transport medium for specimens received by post. Although in one comparison the sodium hydroxide method is slightly more efficient than the trisodium phosphate method, they suggest that the microscopic examination of the specimens treated with

sodium hydroxide was unsatisfactory because of temporary staffing difficulties and that some of these specimens would have been classifed as positive by microscopy by the standards of examination adopted for the trisodium phosphate method. Gifford et al. (1951) and Peizer et al. (1954) found little difference between the two methods but neither of these groups of workers give details of results with specimens negative by microscopy (tables 7 and 8). All of these investigations which show that the trisodium phosphate method is equal to or superior to the sodium hydroxide method were conducted under conditions which differed from those adopted by the Public Health Laboratory Service. Starkey and Aubert used a final concentration of trisodium phosphate equivalent to 3.3 per cent. of the anhydrous salt and Peizer et al. whose study was conducted on specimens obtained from out-patients used 4.3 per cent. of the anhydrous salt as a collection medium (giving a final concentration of approximately 2.5 per cent. trisodium phosphate after the addition of sputum); the final concentration of anhydrous salt used in the Public Health Laboratory Service Trial was 5 per cent. Gifford et al. used 5 per cent. of the anhydrous salt but reduced the exposure time to 10 minutes compared with 24 hours adopted by the Public Health Laboratory Service. These changes might account for the more favourable reports by these workers on trisodium phosphate; it is surprising that their contamination rates (tables 6, 7, and 8) compare favourably with those obtained by the Public Health Laboratory Service.

b) Comparisons between acid methods.

Corper and Uyei (1930) compared oxalic acid with sulphuric acid. They found (table 9) that oxalic acid gave slightly better results than sulphuric acid when used at 5 per cent. strength. On this basis and for reasons of convenience (see p. 9 and p. 10 of this thesis) they recommended the use of 5 per cent. oxalic acid.

Collins (1952) compared the oxalic acid method with acid-iron-peroxide. He found (table 10) that the acid-iron-peroxide method was inferior to the oxalic acid method. This result is in keeping with those of the Public Health Laboratory Service in whose hands acid-iron-peroxide was less efficient than sulphuric acid (table 2).

Collins states that a number of his Löwenstein-Jensen media developed a deep green colour. Not all of these media were contaminated; in some the medium remained acid after inoculation and remained sterile. He suggests that results with acid methods might be improved by neutralising the sediment before inoculating the media. A similar phenomenon was reported by Corper and Cohn (1933a); they found "that the saline washing does not suffice to remove the acid from the tissue residue and that the material when planted may still register a low pH (2-4), an acidity which may possibly persist in the planted specimen for some time after incubation is begun." Neutralisation was not adopted in the sulphuric acid method used by the Public Health Laboratory Service.

Comparisons between alkali methods and acid methods.

Anderson et al. (1953) claimed that, because the concentration of 3 per cent. hydrogen peroxide recommended for the acid-iron-peroxide method by Jungmann (1938) has been misinterpreted, the strength of hydrogen peroxide used by the Public Health Laboratory Service is only one-tenth of the correct strength. They show that, if the method is carried out according to their interpretation of "3 per cent. hydrogen peroxide," liquefaction of sputum is complete in 5 minutes. Although they do not provide results comparable to those provided by the Public Health Laboratory Service, they give results of a small trial with 110 specimens of sputum (table 11) which "support our belief that Jungmann's original method is least not inferior to the alkali method of digestion." Better results might be expected from the acid-iron-peroxide method if the time of exposure to this agent were reduced; the higher concentration of hydrogen peroxide adopted by Anderson et al. shortens the period required for complete homogenisation. Apart from this, the Public Health Laboratory Service (1953) has shown that, in certain centrifuges which tend to heat when operated for prolonged periods, they obtained better results with the acid-iron-peroxide method by reducing the period of centrifugation to 15 minutes instead of the 30 minutes adopted for the 1952 trial (p. 13 of this thesis).

Macfarlane et al. (1955) compared the sodium hydroxide method in two series of specimens. In one series the strength of hydrogen peroxide was that adopted by Anderson et al. (1953) and, in the other, that adopted by the Public Health Laboratory Service for the 1952 trial. The effect of prolonged centrifugation was also taken into account. The best results with the acid-iron-peroxide methods were obtained with a centrifugation period of 15 minutes but even under these conditions neither acid-iron-peroxide method compared favourably with the sodium hydroxide method (tables 12 and 13).

Corper and Uyei (1927) made a comparison between sodium hydroxide and sulphuric acid in 6 specimens of sputum known to contain acid-fast bacilli. They concluded that 6 per cent. sulphuric acid was superior to sodium hydroxide and some of the figures on which they base this conclusion are shown in table 14. In 1930 they suggested that 5 per cent. oxalic acid was superior to 6 per cent. sulphuric acid (see table 9 and pages 9 and 10 of this thesis). These results are rather inadequate but in 1933b Corper and Cohn conducted a trial with specimens in which microscopy failed to demonstrate acid-fast bacilli. Presumably the reason for this trial was that "...it is our experience ..... that results obtained with studies pursued with sputum positive in microscopic smears are of no value in studying methods for isolation, or for studying the value of nutrients, for small numbers of bacilli." In this trial two different sets of workers each succeeded in isolating tubercle bacilli from about 50 per cent. of the specimens by

the oxalic acid method; combining the results of both workers, the proportion of positive results rose to 71 per cent. The results obtained with the sodium hydroxide method are not reported in detail because the contamination rate with this method was very high (about 52 per cent. for egg medium). The work of Corper and his colleagues in this field did much to popularise acid methods of isolating tubercle bacilli from sputum; it must be emphasised that scrutiny of their work suggests that acid methods yielded better results because these methods gave more satisfactory control of contamination and not because acids are less lethal to tubercle bacilli than alkali. In this connection it will be seen that a later (see p. 10 of this thesis) method described by Corper and Stoner (1948) recommends the use of trisodium phosphate - an alkaline salt - for the isolation of tubercle bacilli and that this method failed to give satisfactory control of contamination in the hands of Mullahy or Byham (see p. 17 of this thesis; tables 4 and 5) and that in the 1952 trial by the Public Health Laboratory Service it gave the highest contamination rate of the four methods tested (table 2). Gernez-Rieux, Sevin and Chenet (1949) and Madsen (1950) also compared sodium hydroxide and sulphuric acid. Both found sodium hydroxide the more efficient (tables 15 and 16) but Gernez-Rieux et al. used different series of sputum for each method. Madsen does not separate specimens which are positive on microscopy from those which are negative; the difference in efficiency between the two methods is not very convincing.

In the original paper describing the use of trisodium phosphate, Corper and Stoner (1946) compared the trisodium phosphate method with the oxalic acid method in 18 specimens of sputum which had given negative results by microscopy and by guinea pig inoculation. The trisodium phosphate method yielded 3 positive results whereas the oxalic acid method failed to yield any (table 17). In this small trial, the specimens were exposed to the action of oxalic acid for 1 hour. This is in excess of the exposure time adopted by most laboratories and destroys what little significance these results may have. Van Vranken (1947) also compared trisodium phosphate with oxalic acid and obtained slightly better results with trisodium phosphate (table 18). Because the difference in favour of trisodium phosphate is small and because a different series of specimens was used for each method little significance can be attached to these results. On the other hand Beattie (1949) found hydrochloric acid superior to trisodium phosphate. The majority of her specimens were positive by microscopy and therefore her results probably minimise the difference between the two methods (table 19).

The following conclusions can be made from these comparisons of the results obtained in the trial conducted by the Public Health Laboratory Service and those obtained by other workers who used series of sputum specimens.

1) There is no evidence that any method is more efficient than the sodium hydroxide method. This method should therefore be

used as a control procedure in trials of new methods.

2) The original trisodium phosphate method described by Corper and Stoner (1946) may be modified so that it yields results which compare favourably with those obtained by the sodium hydroxide method. The trisodium phosphate method has a special application in specimens collected from out-patients; it yields its best results when used as a transport medium for these specimens (Peizer et al. 1954; Starkey and Aubert 1950).

3) Acid-iron-peroxide methods do not compare favourably with the sodium hydroxide method or with other acid methods. Even the results of Anderson et al. (1953) who propose further large-scale trials of the method suggest that it is inferior to the sodium hydroxide method for specimens in which acid-fast bacilli cannot be demonstrated by microscopy (table 11).

4) In the trial conducted by the Public Health Laboratory Service, failure to neutralise homogenates prepared by the sulphuric acid method may have biassed the results against this method but it is difficult to assess how far this affected the efficiency of the method in comparison with the other methods.

The lethal effect of decontaminating agents on tubercle bacilli. Quantitative studies. Corper and Stoner (1946) compared the lethal effects on tubercle bacilli of saline, sodium hydroxide, oxalic acid and trisodium phosphate. Aqueous suspensions of tubercle bacilli containing 1, 1×10^{-3}, and 1×10^{-6} mgm. per ml. of organisms were treated with each agent. The results

are presented in table 20 and show that, in a solution of trisodium phosphate with a final concentration of 5 per cent. of the anhydrous salt, tubercle bacilli survive for a longer period than they do in saline. Further, under the conditions adopted for this experiment, exposure to sodium hydroxide or oxalic acid for periods less than 2 hours has a lethal effect on tubercle bacilli and tends to delay the appearance of macroscopic growth from the organisms which survive. A similar experiment was carried out by Spendlove et al. (1949) in which survivalwas assessed by viable counts made at intervals after mixing aqueous suspensions of tubercle bacilli with a number of different agents. These counts were compared with counts obtained by mixing the test suspensions with their respective decontaminating agents neutralised before mixing. Table 21 shows the results obtained with trisodium phosphate, sodium hydroxide and sulphuric acid. There is little difference in the lethal effects of these agents after 40 minutes; after this time there is little increase in the number of organisms killed by trisodium phosphate whereas the number of viable organisms continues to diminish rapidly in the mixture containing sodium hydroxide and even more rapidly in the mixture containing sulphuric acid. Although these results are similar to those of Corper and Stoner (table 20), the method adopted by Spendlove et al. reveals that trisodium phosphate does have

a lethal effect on tubercle bacilli. Spendlove et al. also compared the lethal effects of decontaminating agents on tubercle bacilli in sputum. Ten specimens of sputum were pooled and thoroughly mixed; aliquots of the mixture were then exposed to the action of decontaminating agents and viable counts were made at intervals. The results for sodium hydroxide, trisodium phosphate, oxalic acid and sulphuric acid are presented in table 22. Because the numbers of tubercle bacilli which survived the first periods of treatment were too great to be counted by the method used, no difference in the lethal action of these agents was observed in an exposure time of less than 2 hours. Again the tubercle bacilli survived longest in trisodium phosphate, and longer in sodium hydroxide than in acid.

Gray et al. (1954) also used a viable counting procedure to estimate the lethal effect on tubercle bacilli of procedures used in the routine isolation of these organisms. In a test with a suspension of tubercle bacilli in albumin water, more organisms survived after treatment with sodium hydroxide or trisodium phosphate than after treatment with sulphuric acid, oxalic acid or acid-iron-peroxide (table 23). These results also show that trisodium phosphate has a lethal effect on tubercle bacilli; this is in agreement with the results of Spendlove et al. (table 21). Gray et al. also showed that these decontaminating agents had a similar lethal effect on

tubercle bacilli suspended in purulent, non-tuberculous sputum (table 24) and that the numbers of organisms which could be isolated from tuberculous sputum were greater after alkali treatment than after acid treatment (table 25). The degree of the lethal effect reported by Gray et al. is greater than that reported by Spendlove et al. Although Gray et al. made few observations with all the decontaminating agents, they confirmed the degree of lethal effect of sodium hydroxide by repeated studies on suspensions of different strains of tubercle bacilli in albumin water; the number of organisms which survived was always less than 20 per cent. of the total number exposed. Glover (1952) and Yegian and Budd (1952) also found that sodium hydroxide killed a very high proportion of tubercle bacilli in aqueous suspensions.

The fact that sodium hydroxide is less lethal to tubercle bacilli than acid (Spendlove et al., p. 25 of this thesis; Gray et al., p. 26 of this thesis), agrees well with the results of the Public Health Laboratory Service trial which demonstrated that sodium hydroxide is superior to sulphuric acid for the isolation of tubercle bacilli from sputum. Trisodium phosphate, however, is less lethal to tubercle bacilli than sulphuric acid (Spendlove et al.; Gray et al.) or oxalic acid (Corper and Stoner, p. 24 of this thesis; Spendlove et al.; Gray et al.) but, in the hands of the Public Health Laboratory Service, sulphuric acid is significantly more efficient than

trisodium phosphate for the isolation of tubercle bacilli from sputum (see p. 14 of this thesis). Spendlove et al. and Gray et al. maintain that the toxic effects of decontaminating agents are not minimised by sputum. Gray et al. show that the effect of decontaminating agents on tubercle bacilli suspended in purulent non-tuberculous sputum is the same as that on tubercle bacilli in aqueous suspension. It must be emphasized, however, that neither Spendlove et al. nor Gray et al. present figures showing in absolute terms the lethal effect of decontaminating agents on the tubercle bacilli contained in sputum expectorated by tuberculous patients. Griffith (1916) showed that "thick" sputum protected tubercle bacilli from the lethal effect of antiformin and Yegian and Budd (1952) made a similar observation on the lethal effect of sodium hydroxide; Yegian and Budd, however, express the opinion that the protective effect of sputum is unlikely to operate in specimens containing very small numbers of tubercle bacilli.

Spendlove et al. consider that the difference in the results yielded by quantitative studies and by studies based on series of specimens of sputum may be explained by factors which quantitative methods do not assess; for example, although acid is more lethal to tubercle bacilli than alkali, centrifugation may concentrate tubercle bacilli from an acid-homogenate of sputum more efficiently than from an alkali-homogenate. This explanation, however, throws little light

on the peculiar problems concerning the performance of trisodium phosphate. In quantitative studies it compares favourably with sodium hydroxide but the Public Health Laboratory Service (p. 14 of this thesis), Mullahy (p. 17 of this thesis) and Byham (p. 17 of this thesis) found it inferior to sodium hydroxide. The most promising results with trisodium phosphate were obtained by Starkey and Aubert (p. 17 of this thesis) and by Peizer et al. (p. 18 of this thesis); these workers used a lower concentration of trisodium phosphate than that recommended by Corper and Stoner (p. 24 of this thesis). Gifford et al. (p. 18 of this thesis) also obtained good results with trisodium phosphate; they used a much shorter time of exposure to trisodium phosphate than that recommended by Corper and Stoner.

The following conclusions are reached by comparing quantitative studies with studies conducted with series of sputum specimens: (1) Contradictions between the results yielded by the two methods are difficult to explain satisfactorily; (2) promising results obtained in quantitative studies require to be verified by studies with sputum specimens. This conclusion is the opposite of that made by Corper and Cohn (1933a) who suggest that "observations with routine sputa" are "of little value for comparison of methods or media unless verified by accurate quantitative studies." (3) There are no absolute quantitative results concerning the action of decontaminating agents on tubercle bacilli expectorated by tuberculous patients.

NEW METHODS OF ISOLATING TUBERCLE BACILLI FROM SPUTUM

The isolation of tubercle bacilli from sputum by means of Teepol

Browning et al. (1953) described a simple method of isolating tubercle bacilli from sputum by treating the sputum with an equal volume of Teepol - a proprietary fluid containing 20 to 22 per cent. of sodium secondary alkyl sulphates. After a lapse of 6 or 24 hours, a few loopfuls of the mixture were spread on Löwenstein-Jensen medium. Their results are shown in table 26; the method is very similar to that proposed by Griffith (1914) for antiformin. The method is clearly very satisfactory for specimens known to contain acid-fast bacilli; lack of comparison with other methods prevents any judgement of the efficiency of the method for specimens in which tubercle bacilli cannot be demonstrated by microscopy. Tison and Loze (1954) compared the efficiency of Teepol with that of Tison's (1953) modification of the sodium hydroxide method. The Teepol method used by Tison and Loze differed from that of Browning et al.; the homogenate was diluted with distilled water and centrifuged and the deposit was washed with distilled water by a further period of centrifugation. Each of fifty-five specimens of sputum were divided into equal parts and one part was assigned to each method; 4 Lowenstein-Jensen

slopes were inoculated by each method. The results (table 27) are expressed in terms of the number of slopes positive by each method. The proportion of specimens which were positive by microscopy before culture is not stated. The results suggest that the Teepol method is slightly less efficient than the sodium hydroxide method.

Tison (1954) described the use of Teepol combined with sodium hydroxide. Sputum is treated with a solution containing 25 per cent. Teepol and 1 per cent. sodium hydroxide. Comparison of this method with the sodium hydroxide method showed that it was superior to the sodium hydroxide method (table 28). It is difficult to judge the degree of superiority because the results are expressed in terms of the number of slopes of medium yielding cultures of tubercle bacilli and not in terms of specimens or patients. A similar comparison made by Tison and Audrin (1955) showed that the sodium hydroxide-teepol method was superior to the sodium hydroxide method. The results are presented in table 29; the figures refer to specimens.

Quaternary Ammonium Compounds.

Saxholm (1954) described the isolation of tubercle bacilli from sputum by digesting the sputum with a mixture containing 1 per cent. pancreatin and 1 per cent. Desogen - a proprietary quaternary ammonium compound manufactured by Geigy. The homogenate was inoculated on Löwenstein-Jensen medium without concentration by centrifugation. He

compared this method with the sodium hydroxide method and found that treatment with pancreatin-desogen method for 4 or for 24 hours was more efficient than treatment with sodium hydroxide (tables 30 and 31). Later (1955) he made further comparisons between the two methods. Results obtained with the pancreatin-desogen method were approximately the same as those obtained with the sodium hydroxide method (table 32). Because the contamination rate was high he increased the concentration of Desogen in the pancreatin-desogen mixture to 1.5 per cent. Table 33 shows that the increased concentration of Desogen reduced the contamination rate to a satisfactorily low level without impairing the efficiency of the method for isolating tubercle bacilli.

Hirsch (1954) suggested the use of Zephiran, a proprietary brand of the quaternary ammonium compound benzalkonium chloride for the isolation of tubercle bacilli because, although a concentration of 0.002 per cent. of Zephiran had a bacteriostatic effect on tubercle bacilli, concentrations up to 0.05 per cent. had no bactericidal effect on tubercle bacilli but had a marked bactericidal effect on other micro-organisms. Saxholm (1955) published a comparison of a pancreatin-zephiran method with his pancreatin-desogen method and the sodium hydroxide method. He found that the pancreatin-zephiran method gave poorer results than the pancreatin-desogen method or the sodium hydroxide method, (table 34). The concentration of benzalkonium chloride in the mixture (1 per cent.) might be expected from the results of Hirsch to have a lethal effect on tubercle

bacilli.

The only other observations on the efficiency of the pancreatin-desogen method are those of Lind (1956) who found the sodium hydroxide method better than the pancreatin-desogen method. Table 35 shows that this difference is small despite the very high contamination rate with the pancreatin-desogen method.

Neither Saxholm nor Lind give adequate figures concerning the efficiency of the pancreatin-desogen method for specimens negative for acid-fast bacilli by microscopy. It would therefore be unwise to adopt these methods for routine use unless results with specimens negative for acid-fast bacilli by microscopy show that the method compares favourably with the sodium hydroxide method.

Patterson et al. (1956) recommend the use of a combination of trisodium phosphate with Zepniran for the isolation of tubercle bacilli from sputum. Sputum was mixed with an equal volume of 10 per cent. trisodium phosphate solution containing 0.1 per cent. Zephiran. After 15 minutes the homogenate was centrifuged and the deposit washed by resuspension in 48 ml. of sterile saline and recovered for culture by a second period of centrifugation. This method was compared with the sodium hydroxide method and with the trisodium phosphate method. The results (tables 36 and 37) showed that this method was more efficient than either the sodium hydroxide method or the trisodium phosphate method. Once again there is insufficient information concerning the efficiency of the method of Patterson et al. for specimens known to contain few tubercle bacilli.

THE PLACE OF MICRO-CULTURAL METHODS IN THE ISOLATION OF TUBERCLE BACILLI

Although microscopic methods of detecting and observing the growth of tubercle bacilli have been used since the time of Koch, Pryce (1941) was the first to suggest that such methods might be applied in routine cultures from specimens of sputum. The main advantage of micro-culture methods is that "a single clump of acid-fast bacilli that would be found only by laborious search of a stained film is thereby converted in a few days into a colony that can readily be identified with the 2/3 inch objective." (Whitby and Hynes, 1956). In most of the methods described, sputum is smeared on a slide, the slide is decontaminated with sulphuric acid, washed and incubated in fluid medium. Results may be available in 2 to 6 days and there is no advantage in prolonging the incubation beyond 14 days (Simpson and Reed, 1955). With specimens in which acid-fast bacilli could not be demonstrated by microscopy, Oeding (1951) and Hesselberg and Oeding (1952) found that micro-culture yielded fewer positive results than the sodium hydroxide method (tables 38 and 39); on the other hand, micro-culture gave very good results with specimens known to contain acid-fast bacilli.

Berry and Lowry (1950) also provide figures which appear to confirm those of Oeding and those of Hesselberg and Oeding (table 40). Nevertheless Berry and Lowry "believe that the slide-culture method is more efficient than the figures shown ... would seem to indicate for the following reasons: (1) The number of specimens lost by contamination was high in each method, 9 per cent. for slide culture and 12 per cent. for routine culture. However, apparently by chance, a greater number of positive specimens were contaminated in the slide culture than on routine culture, (2) during the first half of the survey we lost 8 per cent. of the films from the slides .... Because the problem of losing films has been largely solved, and since the contamination rate with this method under normal conditions averages less than 3 per cent., we believe that it is justifiable to present the survey figures with technically unsatisfactory specimens excluded ..... The slide culture was positive in 82 per cent. Similar consideration of the routine method would bring the percentage of positive cultures to 84 per cent." They admit that "the amount of work involved in the slide culture method was undoubtedly greater than that required by the routine methods, not because the actual preparation of the cultures for incubation was more time-consuming, but because of the time required to examine the slides." On the other hand results were obtained sooner with the slide-culture method: "The average time for routine cultures was 37.4 days. As a matter of fact, almost 20 per cent. of the

positive routine cultures showed growth only after 6 weeks or more of incubation. The average time of incubation for the positive slide cultures was three and one-half days .... Even if it proves that, under ideal conditions the slide culture method is not as sensitive as culture on solid medium, many slide cultures from repeated specimens can be completed while one routine culture is being incubated."

Simpson and Reed (1955) evolved an apparatus for the rapid decontamination of slide-culture specimens; preparations from 10 specimens can be decontaminated simultaneously without risk of transferring tubercle bacilli from one specimen to another. They were able to apply the slide-culture method to a large series of routine specimens; as a control procedure they treated part of each specimen with a solution of 25 per cent. trisodium phosphate. Their results are presented in table 41. Despite the fact that they chose a control procedure which failed to yield satisfactory results in the hands of other workers (Public Health Laboratory Service 1952; Mullahy, 1950; Byham, 1950) their results suggest that slide-culture may be applied with davantage in routine work with specimens negative by microscopy. It must be emphasised, however, that the specimens were homogenised in a paint conditioner before culture and that this procedure and the necessity for microscopic examination after culture make heavy demands on the time of the operator. The observations made by Simpson and Reed have been confirmed in later work by these authors (Simpson and Reed 1956).

Chu (1955) described a micro-culture method which is more efficient than culture by the sodium hydroxide method with specimens negative by microscopy. Sputum is homogenised by the sodium hydroxide method, then neutralised with hydrochloric acid. To the neutralised homogenate is added an equal volume of a complex fluid medium containing salts, lecithin, asparagin, glucose, Tween 80, plasma and penicillin. The mixture is incubated for 24 hours and centrifuged, and a smear is made from the deposit. If acid-fast bacilli are not seen in this smear, 14 smears are made on sterile slides; these slides are then incubated in a medium similar to that already described above but omitting Tween 80 and substituting laked blood for plasma. The slides are examined at frequent intervals. Table 42 shows that culture on solid medium from the sodium hydroxide homogenate yielded fewer positive results than the micro-culture method; in addition, the results were available very much sooner by the micro-culture method. A similar, but less complicated method, is described by Tison (1956) but he does not compare this method with any standard method.

Apart from the fact that micro-culture methods require microscopy for the recognition of positive results their adoption as a complementary method to standard method involves the introduction of extra work in the

setting up of cultures. Hoyt et al. (1954) described a micro-culture method which may be incorporated with little additional labour in routine inoculation procedures. Sterile filter paper strips are placed on the surface of the medium used for routine purposes. These media are inoculated in the usual way with homogenised sputum and after 10 days, the strips are mounted on slides, stained by the Zeihl-Neelsen method and examined for micro-colonies. Hoyt et al. found that this method yielded fewer positive results than the standard sodium hydroxide method (table 43). It would be suitable, however, for use as a rapid supplementary method in routine laboratory practice.

Micro-culture methods have also been used for testing the sensitivity of tubercle bacilli to antituberculous drugs (for example, Rubbo and Morris 1951; Rice and Rowan 1953; Collard and Mann 1953; Reed 1954). In these methods the advantage of speed in obtaining results is largely offset by the number of preparations required for each test. For routine tests, most laboratories now use solid media incorporating suitable concentrations of anti-tuberculous drugs; these media may be inoculated with a suspension of tubercle bacilli obtained from a culture or, if a sufficient number of acid-fast bacilli is present in the sputum, from a homogenate of the sputum. With little

additional labour the incorporation of the filter-paper strip method of Hoyt et al. in sensitivity tests made on solid media would have 2 advantages: (1) it would allow early reading of the test and (2) it would permit a review of these early results in the light of those obtained after further incubation of the media until macroscopic growth had appeared. This second procedure is necessary because of the report by Reed (1954) that the results obtained in sensitivity tests by micro-culture occasionally disagree with those obtained by standard methods.

It may be concluded, therefore, that micro-cultural procedures have a clear advantage over standard methods because they yield results very much sooner. For the routine isolation of tubercle bacilli from sputum they involve additional work and in order to obtain satisfactory results from specimens negative by microscopy, a very complicated procedure is required (Chu 1955). Micro-culture methods may have a useful application in routine sensitivity tests; the simplicity of the filter paper method described by Hoyt et al. suggests that its application to sensitivity testing should be examined.

METHODS OF ISOLATING TUBERCLE BACILLI WHEN SPUTUM IS NOT AVAILABLE

The following methods are used in cases of pulmonary tuberculosis when no sputum is available or when examination of sputum has given negative results: gastric lavage, laryngeal swabbing, and tracheo-bronchial lavage. The isolation of tubercle bacilli from specimens obtained by gastric lavage was first described by Meunier(1898) and until 1940 was the standard method used in patients who failed to produce specimens of sputum. In 1940 Nassau recommended the use of laryngeal swabs for this purpose; because this type of specimen can be obtained easily and does not require an elaborate treatment in the laboratory it has become very popular in this country. In 1946 Abreu introduced tracheo-bronchial lavage and claimed that this procedure yielded more positive results than gastric lavage. Two problems arise in the consideration of these methods: (1) Which is the most efficient procedure? (2) Can the laryngeal swab method - the simplest of the three - be used instead of methods based on the examination of sputum for the detection of tubercle bacilli in cases of minimal tuberculosis or in patients who have made a favourable response to treatment?

Nassau's (1940) method of laryngeal swab culture yielded positive results in 63 of 166 patients; these patients

were either unable to produce sputum or, at the time of examination, had sputum from which tubercle bacilli could not be isolated by cultural methods. Unfortunately the method was not compared with gastric lavage. Many later workers found that the laryngeal swab method compared favourably with gastric lavage. Forbes et al. (1948) found no great difference in the efficiency of the two methods in out-patients; however, in hospital patients who had clinical evidence of pulmonary tuberculosis but had either no sputum or had given negative results by sputum culture, gastric lavage gave better results than laryngeal swabbing (table 44). Hounslow and Usher (1948) found that a combination of 3 laryngeal swab investigations gave better results than a single investigation by gastric lavage. Gilje (1948) demonstrated tubercle bacilli in 13.75 per cent. of 400 laryngeal swab cultures but in only 6.2 per cent. of gastric lavage cultures; a later investigation by the same author (1951) yielded similar results. Frostad (1954) compared the number of positive results obtained from 1500 laryngeal swab examinations with the number obtained from 1500 gastric lavage specimens. Each patient who contributed specimens used in this study was examined by both procedures on the same day. He obtained 179 (11.93 per cent.) positive results by the laryngeal swab method and 195 (13 per cent.) by gastric lavage. Both investigations were positive in

only 91 instances and Frostad therefore suggests that every patient should be investigated by both methods.

On the other hand, a number of investigations suggest that laryngeal swabbing is very much less efficient than gastric lavage. For example, Chaves et al. (1953), in an investigation in which laryngeal swabs and gastric contents were examined from each patient, obtained only 9.6 per cent. positive results by the laryngeal swab method compared with 13.2 per cent. of positive results by culture of gastric contents (table 45). Again Tonge and Hughes (1956) in a similar investigation obtained only 11.6 per cent. positive results by laryngeal swab compared with 28 per cent. by gastric lavage (table 46).

These following factors may explain the contradictory findings in comparisons between the results of laryngeal swab culture and culture of gastric contents. Gilje, who found the laryngeal-swab method much more efficient than gastric lavage, cultivated the laryngeal swabs in his own laboratory but sent the gastric lavage specimens to a central laboratory. It has been established by a number of workers (for example, Sprick and Towey, 1946; Holloway and Cummings, 1949; Rambol, 1951) that the number of positive results obtained in the culture of gastric contents is greatly diminished by storage of the specimen before culture. Edwards (1956) presents figures which suggest that storage before culture does not have the same serious effect on laryngeal swab

specimens and Gilje (1951) found that if both laryngeal swabs and gastric-lavage specimens were sent to the central laboratory, the results still favoured the laryngeal swab method. What is clearly shown by Gilje's work is that laryngeal-swab specimens cultured at once yield much better results than gastric lavage specimens which are delayed in transit before culture. Forbes et al. (see p. 41 of this thesis) found that gastric lavage specimens from out-patients yielded only slightly more positive results than laryngeal swabs, whereas gastric lavage specimens from hospital patients yielded a substantially greater number of positive results than laryngeal swabs (table 44). The difference between the two sets of patients may depend on the fact that, in hospital patients, the specimen of gastric contents can be obtained before the patient has had food and before the stomach contents have been altered by the beginning of the day's activities whereas the quality of the specimen obtained from an out-patient may be poorer because (1) he may not be fasting and (2) if he is fasting, the specimen has probably lain in his stomach for some considerable time before it is removed. The poor results obtained with the laryngeal swab method by Chaves et al. and by Tonge and Hughes must not be over-emphasised. Chaves et al. made their study in ".... a large number of clinics with frequently changing personnel inexperienced in the laryngeal swab technique." Apart from this, both groups of workers used alkali for decontaminating the swabs before

culture. Although cultures were made from the swabs and from the fluid with which they were treated it is possible that the liquefaction of the material on the swabs by the alkali allowed most of the tubercle bacilli present to soak far into the swab so that they could not be transferred to the culture medium. Nassau (1940), Forbes et al. (1948), Hounslow and Usher (1948) and Gilje (1948, 1951) all used an acid decontamination method for laryngeal swabs; these are the authors who report favourably on the method.

The results presented by Nassau (1940; see p. 40 of this thesis) suggest that the laryngeal-swab method might yield more positive results than the routine culture of sputum in patients whose sputum contains very few tubercle bacilli. Nassau claims that the material obtained by laryngeal swabbing "may be regarded as concentrated sputum undiluted by saliva and nasopharyngeal secretion." Nevertheless, Forbes et al. (1948) in patients whose sputum was negative for acid-fast bacilli by microscopy, found that sputum culture yielded almost twice as many positive results as laryngeal swab culture (table 47). Hata et al. (1950) compared a number of methods of isolating tubercle bacilli from a group of tuberculous patients. In a series of 130 specimens obtained from these patients, laryngeal swab culture was positive in 16.9 per cent.; the corresponding figure for sputum culture was 37.7 per cent. and

for gastric lavage culture 43.9 per cent. (table 48). Lind and Lundin (1955), in a series of 381 investigations obtained 56 positive results by sputum culture when the corresponding results by laryngeal swab culture were negative; the opposite finding was obtained in only 8 instances.

Tracheal lavage was introduced by Abreu in 1946; his method is as follows. Saline is introduced into the respiratory tree under local anaesthesia and the return fluid expectorated by the patient is cultured for tubercle bacilli. In 18 patients, from each of whom a gastric-lavage specimen and a tracheal-lavage specimen were taken on the same day, he obtained 8 positive results from the tracheal specimens and only 1 from the gastric specimens. Gernez-Rieux, Breton, Delwaulle and Sevin (1949) obtained 151 positive results by tracheal lavage from 310 patients in whom other investigations including gastric lavage were negative. Wardrip et al. (1949) also compared the result obtained by tracheal lavage with those obtained by gastric lavage; in their hands tracheal lavage yielded twice as many positive results as gastric lavage (table 49) and gastric lavage never gave a positive result in a patient in whom tracheal lavage was negative. On the other hand, Deakins and Barber (1953) failed to find a significant difference between the numbers of positive results yielded by gastric lavage and tracheal lavage, (table 50) but it must be emphasised that the tracheal-lavage specimens were treated

with 2 per cent. sodium hydroxide (final concentration) for 2 hours whereas the duration of treatment for gastric lavage specimens was only 15 minutes. The results of Deakins and Barber are therefore biased in favour of the gastric-lavage specimens. Recently Lees et al. (1955) compared the efficiency of tracheo-bronchial lavage, gastric lavage and laryngeal swabbing in 144 patients "in whom minimal, possibly active, pulmonary tuberculosis had been diagnosed as the result of routine radiography." Table 51 shows their results which demonstrate that tracheo-bronchial lavage is easily the most efficient of the three methods tested; it must be emphasized, however, that the application of the 3 methods together yielded much better results than any one of the methods applied alone.

Consideration of methods of isolating tubercle bacilli from patients who are unable to provide sputum specimens leads to the following conclusions.

(1) Laryngeal-swab specimens are more easily obtained than specimens of gastric contents or tracheal-lavage specimens.

(2) It is uncertain whether results obtained from laryngeal-swab culture are as good as those obtained by culture of gastric contents. In comparisons of the laryngeal-swab culture with culture of gastric contents, the results most favourable to the laryngeal swab method have been obtained (a) under circumstances in which cultivation of the gastric contents is

delayed (Gilje 1948, 1951) or (b) in out-patients from whom satisfactory specimens of gastric contents may not always be assured (Forbes et al. 1948).

(3) The available evidence suggests that, if the patient is able to produce sputum, laryngeal swab culture yields results inferior to those obtained by culture of sputum.

(4) Tracheal-lavage specimens yield many more positive results than gastric-lavage specimens. In one investigation (Lees et al. 1955) tracheal-lavage specimens were also superior to laryngeal-swab specimens.

SIMPLICITY AND SAFETY IN LABORATORY PROCEDURES FOR THE DIAGNOSIS, TREATMENT AND CONTROL OF TUBERCULOSIS

Simplicity

All the standard methods of isolating tubercle bacilli from sputum involve centrifugation. This is not a major difficulty when small numbers of specimens are handled but limits the number of specimens which can be handled in one day by a small laboratory. The main purpose of centrifugation is to concentrate the tubercle bacilli; it may also be used to wash the organisms free of a decontaminating agent. If the decontaminating agent is sulphuric acid, oxalic acid, sodium hydroxide or trisodium phosphate, its action may be stopped by neutralisation. Hanks et al. (1938) found that centrifugation of homogenates made from sputum by the sodium hydroxide method produced very poor concentration of the tubercle bacilli in the deposit. They recommended a flocculation procedure for use with the sodium hydroxide method and demonstrated by microscopy that this modification produced a concentration of tubercle bacilli twice as great as that produced by the sodium hydroxide method. On the other hand, Baker (1951) showed that the flocculation method of Hanks et al. compared unfavourably with the sodium hydroxide method for the isolation of tubercle bacilli from specimens negative by microscopy.

Klein et al. (1952) also investigated the efficiency of centrifugation and concentrating tubercle bacilli from homogenates of sputum produced by sodium hydroxide. By means of a viable-counting method they showed that the number of organisms per unit volume in the deposit after centrifugation was greater then the number in the homogenate before centrifugation; both of these numbers exceeded the number present in the superficial layers of the supernatant fluid after centrifugation. Nevertheless, in a series of 409 specimens of sputum submitted for routine examination, they obtained 98 positive results by culturing the homogenate before centrifugation compared with 100 positive results after centrifugation. They conclude that "centrifugation of digested specimens at 3000 r.p.m. for 15 minutes is not a highly efficient method of concentrating tubercle bacilli. Examination of the supernatant fluid, by culture or animal inoculation, should not be neglected." Hata et al. (1950) also provide figures which suggest the same conclusion (table 48) and Saxholm's (1954, 1955) pancreatin-dæsogen method which does not involve centrifugation yielded results which compare favourably with those of a sodium hydroxide method in which the homogenate was concentrated by centrifugation (tables 30 to 34). Unfortunately, none of the studies which suggest that centrifugation adds little to the

efficiency of standard methods of isolating tubercle bacilli from sputum give results based on specimens negative by microscopy. Such a study is required in order to establish whether or not centrifugation is really essential for the efficient operation of these methods.

Of the new methods of isolating tubercle bacilli from sputum, that of Browning et al. (1953; see p. 30 of this thesis) and that of Saxholm (1954, 1955, see p. 31 of this thesis) do not involve centrifugation; they have the additional advantage that prolonged exposure to the agents employed has little effect on their efficiency. This last advantage also applies to the trisodium phosphate method (see p. 10 of this thesis) and is important when dealing with large numbers of specimens; it also permits the use of these agents as transport media for specimens from out-patients. The Teepol method described by Browning et al. has not been compared with any standard method in specimens negative by microscopy; the modifications suggested by Tison and Loze (1954) and Tison (1954) involve centrifugation and, although Tison's (1954) method compares favourably with the sodium hydroxide method it offers no advantage in simplicity.

Micro-culture methods are more complicated than standard methods. Even if a simple procedure is used for the setting up of cultures - for example, the use of special apparatus for the simultaneous decontamination

of preparations from 10 specimens (Simpson and Reed, see p. 36 of this thesis) or the use of filter paper strips on the surface of solid medium (Hoyt et al., see p. 38 of this thesis) - the need for microscopic examination at the end of the incubation period cannot be eliminated. For this reason and because the introduction of an additional procedure requires more space and more technical assistance, these methods are less popular than they deserve to be; for example, Hesselberg and Oeding (1952) reluctantly abandoned the use of micro-cultural methods for lack of space and lack of skilled technicians.

Laryngeal-swab culture is the simplest procedure for the isolation of tubercle bacilli from patients who fail to produce sputum. This method is less efficient than tracheal lavage or gastric lavage (Lees et al., 1955, see p. 46 of this thesis); on the other hand, favourable results have been obtained with the laryngeal-swab method in out-patients (Forbes et al., p. 45 of this thesis). From the point of view of laboratory procedure, the simplicity of swab culture is very attractive. Nassau (1954) describes the application of this method to sputum culture but figures provided by O'Hea (1957; tables 74 to 79 of this thesis) show that, although the method is satisfactory for specimens known from microscopy to contain acid-fast bacilli, it is significantly inferior to the sodium-hydroxide method in specimens which are negative for acid-fast bacilli by

microscopy. The results of Forbes et al. (see p. 44 and table 47) suggest that, in patients who can provide specimens of sputum, even laryngeal-swab culture will fail to yield results which compare favourably with those obtained by standard methods of sputum culture.

Under present conditions in this country, the efficiency of laboratory procedures applied to specimens from tuberculous patients depends to a great extent on the ease with which these procedures may be applied to large numbers of specimens. Two simple methods of isolating tubercle bacilli from sputum might be sufficiently sensitive for routine use; (1) Saxholm's pancreatin-desogen method and (2) the cultivation of the neutralised homogenate produced by the sodium hydroxide method, omitting concentration by centrifugation. The efficiency of these procedures should be investigated using specimens in which acid-fast bacilli cannot be demonstrated by microscopy.

Simplicity in methods of isolating tubercle bacilli is also important from the point of view of safety; the fewer dangerous manipulations involved and the smaller the degree of skill required, the greater the chance that the method may be applied on a large scale without breach of the rules for the safe operation of a tuberculosis laboratory.

Laboratory workers who handle tuberculous material on a large scale are exposed to the risk of contracting tuberculosis. It is not easy to obtain figures which indicate the degree of risk but, under the auspices of the National Association for the Prevention of Tuberculosis, a special study has been carried out during the past four years by Dr. Donald Reid, Reader in the London School of Hygiene and Tropical Medicine. I am indebted to him for permission to quote the results set out in table 52. It is shown that laboratory technical staff have a rate of infection with tubercle bacilli 2 to 4 times greater than that experienced by the general population. Table 52 is only a summary of Reid's figures; his analysis of the material in age groups makes it clear that the danger ages for pathologists lie between 35 and 44 years and for technicians, between 15 and 24 years. A detailed account of this study has recently been published (Reid, 1957).

The following additional observations have been made. "The hazards of accidental infection among laboratory workers are becoming a matter of constantly greater concern to laboratory directors" (Fish and Spendlove, 1950). "While our information on the incidence of laboratory infections is too meagre to have great validity, individual instances of tuberculosis contracted in laboratories which employ non-tuberculous personnel are known in sufficient numbers to cause concern" (Anderson, 1950). ".... Tuberculosis morbidity rates are considerably higher for student nurses, laboratory workers and medical students than for other hospital personnel not/

having similar exposure .... and considerably higher than for young adults not working in a hospital environment" (Riggins, 1953). Sulkin and Pike (1951a, 1951b) give information about a number of different laboratory infections including tuberculosis. They emphasise that the incidence of laboratory infections is very high in persons carrying out diagnostic procedures (table 53).

There are several possible mechanisms of laboratory infection from tuberculous material. Recently, however, great emphasis has been laid on the danger of infection by tubercle bacilli released into the atmosphere in the form of an aerosol. Long (1951) states: ".... probably more emphasis than usual should be laid upon measures to prevent contamination of the air. The possibilities of such contamination are abundant." Evidence supporting this view has been provided by the work of Anderson et al. (1952). Using cultures of Serratia indica they demonstrated that many laboratory procedures liberate micro-organisms in the form of an aerosol and that large numbers of organisms are dispersed into the atmosphere by methods which involve splashing or bubbling. Safety measures recommended for tuberculosis laboratories must therefore include some method of dealing with dangerous aerosols. Wedum, quoted by Anderson (1950) considers "that the most important single item in a laboratory

safety programme is the provision of adequate bacteriological transfer cabinets properly equipped with ventilating devices so that aerosols will be swept away." This kind of apparatus has been recommended by many others; for example, Fish and Spendlove (1950), Gray and Mattinson (1952), Solotorovsky et al. (1953), Jensen (1954). Some of these workers make use of ultra-violet irradiation in their safety hoods but although Wells and Lurie (1941) demonstrated that ultra-violet irradiation prevented cross-infection by the tubercle bacillus in rabbits, Hobby and Lenert (1955) believe that ultra-violet irradiation cannot protect laboratory workers from the immediate danger of accidents in which large numbers of tubercle bacilli are released into the atmosphere. In addition to other precautions Gray and Mattinson recommend the use of masks to prevent laboratory infection. In this connection it is interesting to note that Lurie and Abramson (1949) demonstrated that efficient gauze masks (3 or 6 layers of gauze with 40 x 44 threads per square inch) gave considerable protection to rabbits exposed to aerosols containing virulent bovine tubercle bacilli. They found that masked rabbits had "a 90 to 95 per cent. reduction in the incidence of primary pulmonary tuberculous foci which develop within 5 weeks ..... Indeed 12 of 20 masked animals were completely protected against airborne contagion of such intensity that from 29 to 1027 tubercle bacilli units were deposited in the lungs of simultaneously exposed unmasked rabbits." Lurie and Abramson

are extremely guarded about the possibility of protecting human beings by masks and state "... if the frame into which the mask fitted could be constructed of pliable material which could be accurately applied to the contour of the individual's face around the nose and mouth, and if this contact could be made air-tight there is no reason to believe that the masks could not effectually filter out the dangerous invisible particles that are concerned with the inception of pulmonary tuberculosis."

The measures indicated above have an application in all laboratories dealing with tuberculous material because, no matter how carefully this material is manipulated, at least a few tubercle bacilli may be released into the atmosphere. As a leading article in the Lancet (1952) suggests "Inoculation hoods should be used more widely ..." The same article states that "many laboratory infections are seen in retrospect to have been avoidable." Anderson (1950) expresses the opinion "that the use of techniques which avoid heavy exposure will undoubtedly reduce the incidence of laboratory infection." It would be profitable, therefore, to examine from the point of view of safety common procedures adopted in the handling of tuberculous material. As Gray and Mattinson point out, "any laboratory manipulation of materials containing living tubercle bacilli invariably involves a considerable degree of risk, but through common usage some procedures have come to be regarded

as less dangerous than others."

The dangers of methods of isolating tubercle bacilli. Two operations common to many methods of isolating tubercle bacilli from sputum are particularly likely to release dangerous aerosols: (1) the use of shaking in order to homogenise specimens containing large numbers of tubercle bacilli; and (2) the centrifugation of homogenates prepared from such specimens.

Shaking: This procedure is widely recommended to ensure the efficiency of the common acid or alkali methods of homogenising and decontaminating sputum. Recently mechanical shaking devices have become very popular and a Committee of the American Trudeau Society (1946) states: "the Committee on Evaluation of Laboratory Procedures has voted to recommend more widespread adoption of shaking machines for the homogenisation of sputum for cultures in the belief that there will result not only a great saving of human time and effort, but also that the quality of work will be improved." The use of this kind of apparatus will surely "result not only in a great saving in human time and effort" but also in the production of very dangerous aerosols from sputum containing large numbers of tubercle bacilli; the need for this kind of apparatus is probably restricted to specimens negative for tubercle bacilli by microscopy but the statement by the committee does not make this clear. Moreover, Collins (1951) found that, in comparison

with shaking by hand, shaking in a machine, although it reduced the incidence of contamination with the oxalic acid method from 8.9 to 5.3 per cent., yielded only a slight increase in the efficiency with which tubercle bacilli were isolated (table 54). It must be emphasised, however, that the two procedures were not evaluated with the same specimens of sputum; for this reason the results are suggestive rather than conclusive.

Centrifugation: At first sight this seems an unlikely source of danger from aerosols because homogenates are centrifuged in screw-capped containers. There are two reasons why this operation is less safe than might be imagined. First, a small amount of homogenate may reach the upper part of the outside of the container during shaking, incubation or when the supernatant fluid is decanted when washing homogenised material; this material on the outside of the container may easily be converted into an aerosol during centrifugation. Second, the container may be smashed in the centrifuge. Smashing of the container is a rare event but if heavily infected material is present, it will certainly release a very dangerous aerosol. The dangers of aerosol production by centrifugation of screw-capped containers has recently been studied in detail by Whitwell et al. (1957). They used Serratia as an indicator organism and demonstrated very convincingly that the use of screw-capped containers does not prevent the release of

aerosols during centrifugation.

Safety is seldom mentioned in papers which describe or assess methods of isolating tubercle bacilli from sputum. Gray and Mattinson (1952) are the only workers who make a critical assessment of the possible dangers of a new procedure. In their investigation of the possibility of isolating tubercle bacilli by the intranasal inoculation of mice with tuberculous material, they examined the incidence of cross-infection in laboratory animals and assessed the risk to the worker operating the procedure. They found (1) that mice inoculated intranasally with tuberculous material could transmit infection to uninoculated mice housed in the same cage; (2) that cross-infection could be demonstrated in healthy mice housed close to guinea pigs infected with tubercle bacilli and (3) that "intranasal inoculation, properly performed, is not likely to increase the already present risk to the operators and attendants." They emphasise the need for special safety precautions in dealing with tuberculous material (see p. 55 of this thesis). Again, Jensen's (1954) review of procedures used in tuberculosis laboratories is one of the few which makes it clear that those who operate these procedures must be adequately protected; not only does he lay down safety rules for laboratory staff but he sees fit to include a photographic illustration of a bench equipped with a properly ventilated inoculation hood.

Most of the others who write about routine procedures

to be adopted in dealing with tuberculous material seem to be unaware of the dangers of aerosol production. In the introduction to his survey of methods of demonstrating tubercle bacilli in sputum, Baker (1951) states: "In a laboratory where twenty to thirty concentrations and cultures have to be carried out each day, in addition to direct examinations, a simple method of concentration is necessary, so that a relatively junior technician can be "safely" left to carry out the initial techniques. It was with this view in mind that this survey was initiated." All the methods he tested involve the use of a centrifuge; all except the acid-iron-peroxide method (see below) involve shaking tuberculous material. He does not use the word "safety" elsewhere in his paper and it must be concluded that, in common with most workers in this field, he is so anxious to ensure the efficiency of the methods that he overlooks the possibility that they may be dangerous to those who operate them.

Again Anderson et al. (1956), in a critical article "Use and abuse of Jungmann's method for the liquefaction of tuberculous sputa," emphasise that "those who have worked with the technique (Jungmann's acid-iron-peroxide method) in this laboratory have found sputa easier and cleaner to handle than with other methods, and therefore a wider margin of safety is provided when dealing with larger numbers of specimens." Although the acid-iron-peroxide method does not involve shaking, Anderson et al. seem unaware that a dangerous aerosol may be created by the effervescent

homogenate produced when this method is used with sputum containing large numbers of viable tubercle bacilli.

In an evaluation of his pancreatin-desogen method, Saxholm (1954) states: "It seems that the risk of laboratory infection would be reduced with this method because fewer manipulations are required." This seems incontestable but in describing the method he states: ".....the liquefied contents were sucked up in a pasteur pipette and blown out several times ...."; the likelihood of creating dangerous aerosols by such a procedure is demonstrated by Anderson et al. (1952) and must be taken into account in assessing the safety of this method.

The production of dangerous aerosols by the common methods of isolating tubercle bacilli depends on the numbers of tubercle bacilli in the material examined. O'Hea (1957) suggests that the risks attached to these methods would be reduced by restricting their use to specimens in which acid-fast bacilli cannot be demonstrated by microscopy. He demonstrated that, with specimens containing sufficient tubercle bacilli to give positive results by microscopy, over 90 per cent. of positive results may be obtained by the simple swab-culture method described by Nassau in 1954 (see table 74 of this thesis); this method avoids the danger of aerosol production by homogenisation and centrifugation.

The danger of sensitivity tests. The Medical Research Council

(1953a) recommended the use of Tween-albumin fluid medium for testing the sensitivity of tubercle bacilli to streptomycin and para-aminosalicylic acid. Two qualities of this medium increase the likelihood of exposing the operator to aerosols containing large numbers of tubercle bacilli: (1) it produces a heavy, well-dispersed growth of tubercle bacilli; (2) it has a low surface tension favouring the creation of bubbles during manipulation. Nassau (1954) states: "The use of liquid media for sensitivity testing has been superseded by solid media employed for routine culture. The handling of liquid cultures requires considerable skill, and, even then, is not without danger to the operator." Although the use of solid media for sensitivity tests may be safer than the use of liquid media, the preparation of the inoculum for solid media may produce a dangerous aerosol. For example, in a method described by Nassau (1954), "a standard loopful of the growth is rubbed up in a Wassermann or other sterile tube with 0.5 ml. sterile distilled water." This procedure too "is not without danger to the operator."

Therefore, in addition to general measures for the protection of the operator, attention should be paid to the possibility of minimising the production of dangerous aerosols in the preparation of inocula for sensitivity tests. Two possible methods of achieving this should be examined. First, in tests made directly from sputum, the inoculum might be prepared by Saxholm's method suitably modified in order to

avoid the violent expulsion of tuberculous material from a pasteur pipette; alternatively, a neutralised sodium hydroxide homogenate, prepared without violent agitation of the tuberculous material, could be used for this purpose without preliminary centrifugation. Second, if sensitivity tests have to be made from cultures of tubercle bacilli, emulsification of the primary growth should be avoided; it might be safer to inoculate sensitivity test media from the condensation water or from a suitable fluid medium in contact with the primary growth. Encouraging preliminary results with such a method have been obtained by McNaught (in press).

Conclusions.

(1) There is a need for simplicity in methods of isolating tubercle bacilli not only in order to allow large numbers of specimens to be handled conveniently and efficiently but also to permit the operator to pay due attention to factors which make for safety in his work. Existing methods might be applied without great loss of efficiency by omitting centrifugation.

(2) Reid (1957) confirmed the general belief that it is dangerous to handle tuberculous material. In measures designed to minimise the danger, emphasis is placed on the use of apparatus designed to remove dangerous aerosols from the immediate vicinity.

(3) Despite recent emphasis on the danger of infection from aerosols, very little attention has been paid to this point

in the assessment of methods for the routine isolation of tubercle bacilli from sputum.

(4) Standard methods of sensitivity testing are particularly dangerous; safer methods are needed; existing methods should only be operated in a properly ventilated safety hood.

GENERAL CONCLUSIONS FROM REVIEW OF LITERATURE

(1) It is possible to obtain statistically significant results concerning the efficiency of methods of isolating tubercle bacilli from sputum by comparing different methods using specimens of sputum likely to contain few tubercle bacilli. The conclusions reached in a large trial of this kind by the Public Health Laboratory Service may be reconciled to some extent with contradictory results obtained by other workers in less satisfactory trials.

(2) Trials with specimens of sputum establish only which of the existing methods is most efficient; they are unable to measure the efficiency of a given method in absolute terms. It is therefore impossible to decide from these trials whether a further search for more efficient methods is worth while.

(3) Quantitative studies of methods of isolating tubercle bacilli from sputum suggest that most methods kill at least half of the viable tubercle bacilli treated. Nevertheless, one of the least lethal methods as judged by quantitative studies is the least efficient for the isolation of tubercle bacilli from a series of specimens of sputum in a well controlled comparison with other methods. Quantitative studies of a method must therefore be supplemented with trials of the method in comparison with some standard method in a series of specimens of sputum.

(4) Of the methods recently described for the isolation of tubercle bacilli from sputum, Saxholm's pancreatin-desogen method deserves further trial because it is simple and is apparently as efficient as the sodium hydroxide method — the most efficient of the 4 methods evaluated in the Public Health Laboratory Service trial.

(5) Micro-cultural methods of isolating tubercle bacilli from sputum give much earlier results than standard methods. Under certain conditions they may be as efficient as or more efficient than standard methods. For their routine application they require extra space and greater numbers of skilled workers for their efficient operation. A simple method of micro-culture might usefully be applied without much extra labour in tests of the sensitivity of tubercle bacilli to anti-tuberculous drugs.

(6) Laryngeal swab culture is the simplest procedure for isolating tubercle bacilli from tuberculous patients who are unable to provide sputum. Further study of this method is desirable in order to establish whether it might be generally adopted in patients who either are unable to produce sputum or whose sputum has been examined with negative results.

(7) Existing methods of testing the sensitivity of tubercle bacilli to antituberculous drugs expose the operator to aerosols containing large numbers of tubercle bacilli.

(8) Methods of isolating tubercle bacilli are dangerous to the operator. This danger has not been fully appreciated by many of the workers in this field because of the emphasis placed on efficiency of these methods to the exclusion of other important features and because those who operate these methods are either unfamiliar with the danger of the methods or have operated them over such a long period that they have come to disregard the danger.

(9) Simplification of laboratory procedures would lessen their danger to the operator but it is necessary to provide other measures for the safety of the laboratory worker because it cannot be guaranteed that any manipulation of tuberculous material will not release tubercle bacilli into the atmosphere. For this reason properly hooded and ventilated benches should be provided, especially for those who carry out sensitivity tests.

EXPERIMENTAL WORK

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SCOPE OF THE EXPERIMENTAL WORK

The following are the main objects of the experiments described in this thesis:-

(1) To improve standard methods of isolating tubercle bacilli from sputum from the points of view of efficiency, simplicity and safety.

(2) To establish whether recently described methods of isolating tubercle bacilli from sputum are simpler, safer or more efficient than standard methods.

(3) To evolve a method of isolating tubercle bacilli from sputum which does not involve centrifugation - a dangerous and time-consuming procedure common to all the standard methods. Many of these experiments therefore involve an assessment of efficiency, simplicity and safety.

Assessment of efficiency: Two main methods are available for measuring efficiency:-

(1) Studies made by comparing the numbers of viable tubercle bacilli which survive the application of different methods to portions of a specimen of sputum.

(2) Comparisons of the numbers of positive results obtained by different methods in series of specimens of sputum.

Assessment of simplicity: This is not an experimental

procedure because "simplicity" cannot be expressed numerically. Simplicity is therefore assessed by estimating whether large numbers of specimens of sputum may be handled conveniently by a given method under routine laboratory conditions.

Assessment of safety: This important criterion cannot readily be assessed numerically. It would be a formidable task to estimate the numbers of tubercle bacilli released into the atmosphere by a given procedure. The only experimental assessment of safety which demonstrates the possibility of infection with tubercle bacilli as a result of manipulating tuberculous material is that of Gray and Mattinson (1952) described on p. 59 of this thesis.

On the other hand, there is good indirect evidence concerning the safety of laboratory procedures. By means of a test organism, Serratia indica, Anderson et al. (see p. 54 of this thesis) have shown that laboratory procedures, especially those which involve shaking or bubbling, release micro-organisms into the atmosphere; in a similar way Whitwell et al. (see p. 58 of this thesis) have shown that the centrifugation of material in screw-capped containers may release micro-organisms into the atmosphere. For the purposes of this thesis, therefore, it is reasonable to assume that, with specimens containing large numbers of tubercle bacilli, methods of preparing sputum for culture which involve splashing

bubbling or centrifugation are particularly dangerous.

The experiments in this thesis are arranged in two main groups according to the method adopted for assessing efficiency. These groups are:-

A. Quantitative studies based on counts of viable tubercle bacilli.

B. Studies made with series of specimens of sputum.

The factors of simplicity and safety are commented upon in those experiments in which they are important criteria.

MATERIALS AND METHODS - GENERAL

Materials

All reagents used in the experimental work described in this thesis with the exception of the materials listed below, were obtained from British Drug Houses Limited. The analytical grade of purity was selected for those materials in which this grade is available.

Material	Synonyms	Source	Grade
Ethyl violet	...	Gurr, London	04810
Malachite green	...	Gurr, London	08615
Proflavine sulphate	...	...	B.P.
Penicillin	...	Glaxo Limited	Sodium salt
Polymyxin B sulphate	Aerosporin	Burroughs Wellcome & Co. Limited	...
Bacitracin	...	Sharp & Dohme Limited	...
Mycostatin	Nystatin	Squibb Ltd.	...
Hibitane	Bis-p-chlor-phenyldiguan-idohexane acetate	I.C.I. Ltd.	...
Desogen	Methyl-dodecyl-trimethyl ammonium methosulphate	Geigy Ltd.	10 per cent. commercial solution
Benzalkonium chloride	Alkyldimethyl-benzyl-ammonium chloride Roccal	Bayer Ltd.	10 per cent. commercial solution

Material	Synonyms	Source	Grade
Teepol	Sodium secondary alkyl sulphates	Shell Chemicals Limited	20 per cent. commercial solution
Barium sulphate	...	...	B.P.

Media

Löwenstein-Jensen Medium. This was made according to the formula given by Jensen (1932).

Proskauer and Beck Medium. This is a simple synthetic medium for the growth of tubercle bacilli. The modification described by the American Trudeau Society (1950) was used. It has the following composition:-

Asparagin	0.5 per cent.
KH_2PO_4	0.5 per cent.
K_2SO_4	0.05 per cent.
Glycerol	2.0 per cent.
Magnesium citrate	0.15 per cent.

The ingredients are dissolved in double glass-distilled water in the order given above. After the addition of glycerol, the pH is adjusted to 7 with 40 per cent. sodium hydroxide and then the magnesium citrate is added. The medium is sterilised by autoclaving at 120°C for 15 minutes.

Ethyl violet medium (E.V.) This medium was used in experiment 11 p. 155 of this thesis. The medium has the following composition.

Proskauer and Beck medium containing	
Sterile horse serum	10 per cent.
Ethyl violet	0.001 per cent.
Penicillin	100 units per ml.
Polymyxin	60 units per ml.
Bacitracin	25 units per ml.
Mycostatin	30 units per ml.

The ethyl violet was added to the Proskauer and Beck medium before autoclaving; the remaining reagents were added aseptically from sterile stock solutions after the Proskauer and Beck medium was autoclaved.

Proflavine sulphate medium (P.F.) The use of this medium is described in experiment 11 p. 155 of this thesis. The composition and preparation of the medium are the same as described above for E.V. medium except that proflavine sulphate, 0.0004 per cent. is substituted for ethyl violet.

Methods of preparing sputum for culture.

The sodium hydroxide method.

(a) Homogenisation and decontamination. Sputum was mixed with an equal volume of 4 per cent. sodium hydroxide in a 25 ml. screw-capped container. The mixture was shaken thoroughly by hand and incubated in a water bath at 37°C for 20 minutes. Shaking was repeated after 10 minutes and at the end of the incubation period.

(b) First washing and concentration. The volume of the homogenate was made up to 20 ml. with sterile distilled water and centrifuged for 20 minutes at 3000 r.p.m.

(c) Second washing, concentration and inoculation. The supernatant was discarded and the sediment resuspended in 20 ml. of sterile distilled water. This second washing of the deposit with distilled water was substituted for neutralisation with strong acid because it was shown (see experiment 3, p. 130 of this thesis) that the unintentional addition of even a small excess of acid may reduce the number of viable tubercle bacilli. After centrifuging for a further 20 minutes the supernatant was discarded. The sediment was taken up in a sterile pasteur pipette and from it 2 slopes of Löwenstein Jensen medium were inoculated and a film was prepared for microscopy. The film was stained by the Ziehl-Neelsen method.

The sulphuric acid method.

(a) Homogenisation and decontamination. Sputum was mixed with an equal volume of 3 per cent. (V/V) sulphuric acid in a 25 ml. screw-capped container. The mixture was shaken thoroughly by hand and incubated in a water bath at 37°C for 20 minutes. Shaking was repeated after 10 minutes and at the end of the incubation period.

(b) Washing and concentration. The volume of the homogenate was made up to 20 ml. with sterile distilled water and centrifuged for 20 minutes at 3000 r.p.m.

(c) Neutralisation and inoculation. The supernatant was discarded; the sediment was suspended in 0.5 ml. of sterile

sodium citrate solution and from it 2 Löwenstein-Jensen slopes were inoculated by means of a pasteur pipette.

This method differs in one detail from that used by the Public Health Laboratory Service (1952) - the sediment is neutralised with sodium citrate before transfer to the culture medium. This modification was adopted because of the observation of Collins (see p.19 of this thesis) that, with acid homogenates, a low pH may persist in the culture medium for a prolonged period after inoculation.

The acid-iron-peroxide method. Two reagents are required for this method.

Reagent A: This reagent has the following composition.

Ferrous sulphate	20 gm.
Concentrated sulphuric acid	20 ml.
Distilled water	180 ml.

This reagent keeps indefinitely and may be prepared in bulk.

Reagent B: This reagent consists of 1 volume strength hydrogen peroxide. It must be freshly prepared from 20 volume strength hydrogen peroxide each time the method is applied.

(a) Homogenisation. To 2 ml. of sputum in a 25 ml. screw-capped container are added 1.2 ml. of reagent A and 1.2 ml. of reagent B. The mixture is allowed to stand for 5 minutes at room temperature.

(b) Washing and concentration. The volume of the homogenate is made up to 20 ml. with sterile distilled water and the diluted homogenate is centrifuged for 20 minutes at 3000 r.p.m.

(c) Neutralisation and inoculation. This procedure is exactly the same as described above in step (c) of the sulphuric acid method.

Despite the preference of Anderson et al. (1953) for a concentration of 10 volume strength hydrogen peroxide, a strength of 1 volume was adopted. There are two reasons for this. First, Anderson et al. failed to show that their method was more efficient than the sodium hydroxide method (see p.20 and table 11 of this thesis). Second, Macfarlane et al. provide results which suggest that the acid-iron-peroxide method using 1 volume strength hydrogen peroxide is more efficient than the method described by Anderson et al. (see p. 21 and tables 12 and 13 of this thesis).

The trisodium phosphate method.

(a) Homogenisation. Sputum was mixed with an equal volume of a 10 per cent. solution of trisodium phosphate (anhydrous salt) in a 25 ml. screw-capped container. The mixture was shaken thoroughly by hand and was then incubated overnight at 37°C.

(b) First washing and concentration. The volume of the mixture was made up to 20 ml. with sterile distilled water and centrifuged for 20 minutes at 3000 r.p.m.

(c) Second washing and concentration. The supernatant was discarded and the sediment resuspended in 20 ml. of sterile

water. The deposit obtained by centrifuging this second suspension was used to inoculate 2 slopes of Löwenstein-Jensen medium.

Assessment of the efficiency of methods of isolating tubercle bacilli from sputum

Studies based on counts of viable tubercle bacilli. The method of viable counting used in these studies was that of O'Hea (1955). The procedure is as follows.

Volumes of 4 ml. of Löwenstein-Jensen medium were dispensed in screw-capped bijoux bottles (5 ml. capacity, circular cross section) and inspissated with the bottles in the upright position. The bottles of medium were packed in wire baskets for inspissation and care was taken to ensure that the surfaces of the medium were flat. Satisfactory surfaces were obtained by not overcrowding the bottles near the sides of the baskets. During inspissation and re-heating water of condensation was not formed, but a very small amount sometimes appeared during incubation (fig. 1). This was dealt with as described below. The surface of the medium formed a shallow cup with an area of 1.8 sq.cm. and the depth of the medium is 2 cm. (fig. 1).

To count the number of viable units in a suspension of tubercle bacilli, a series of ten-fold dilutions was made from the suspension in the medium of Proskauer and Beck. A

separate pipette must be used for making each dilution. From a pipette calibrated to deliver drops of 0.02 ml. volume, a single drop from each dilution was placed on the surface of the medium in each of 6 bottles, this number of replicates being sufficient for practical purposes in place of 6. The inoculations were made in series from the greatest to the smallest dilution, and the whole process of inoculating the bottles of medium was done with a single calibrated pipette. After inoculation the bottles were incubated overnight in the upright position at 37°C with screw-caps loose. The caps were then screwed tight to avoid drying of the medium, which may be caused by an imperfect air-seal. Because a trace of condensation moisture was sometimes formed (fig. 1), the bottles were placed in the inverted position for the remainder of the incubation period to avoid possible wetting of the inoculated surface. The incubation period was from 21 to 28 days but a shorter period may suffice for strains such as H37Rv, which have been maintained on artificial media for a prolonged period. After incubation the bottles of medium containing isolated colonies were selected and the number of colonies in each was counted with the aid of a hand lens. The colonies are readily visible (figs. 1 and 2).

The advantages of this method are: the prepared medium and the Proskauer and Beck diluent can be stored ready

for use; airborne contamination is so infrequent as to be negligible; and the accuracy of the method is at least sufficient for comparative purposes.

Studies based on series of specimens of sputum

Specimens of sputum. Sputum was obtained from in-patients of the tuberculosis division of Ruchill Hospital, Glasgow; all the patients from whom sputum was obtained were known cases of pulmonary tuberculosis. The great majority of the patients were receiving streptomycin, para-aminosalicylic acid, isoniazid or a combination of these drugs. The influence of the specific treatment of the patients on the results of cultures for tubercle bacilli made from their sputum cannot readily be assessed. It may well be important but it was not feasible to restrict the studies presented in this thesis to specimens of sputum obtained from the very small numbers of patients who were not receiving specific therapy.

Selection of specimens of sputum. Sputum can readily be classified into 2 main types of specimen by microscopy: (1) specimens in which acid-fast bacilli can readily be detected and (2) specimens in which acid-fast bacilli cannot readily be detected. Both types of specimen may yield valuable information; specimens of the first type usually contain large numbers of viable tubercle bacilli and have been

selected for preliminary tests of methods of isolating tubercle bacilli from sputum; in a group of specimens of the second type there will be some which contain only a few viable tubercle bacilli and others which do not contain viable tubercle bacilli. The second type of specimen has been selected for critical tests of methods of isolating tubercle bacilli from sputum.

Preliminary tests of methods of isolating tubercle bacilli from sputum. Specimens of sputum containing large numbers of viable tubercle bacilli were used. The application of a method of isolating tubercle bacilli from sputum to this type of specimen yielded the following information. First, it demonstrated whether tubercle bacilli could survive the proposed method and yield a culture. Second, the time taken for colonies to appear and the abundance or scarcity of the numbers of colonies indicated whether the method might compare favourably with the standard sodium hydroxide method in a more critical trial; on Löwenstein-Jensen medium the sodium hydroxide method usually yields within 25 days a confluent growth of tubercle bacilli from specimens shown by microscopy to contain numerous acid-fast bacilli. Third, a rough indication was obtained of the ability of the proposed method to control the growth of contaminants.

The application of the preliminary testing procedure described above made it possible to test a large number of methods and to select the few methods worthy of the more

critical and time-consuming procedure described below.

Critical tests of methods of isolating tubercle bacilli from sputum

Critical assessment of the efficiency of a proposed method of isolating tubercle bacilli was made by comparing the efficiency of the method with that of the sodium hydroxide method in a carefully selected group of specimens.

Selection of specimens. Specimens of sputum were examined from a group of patients suffering from pulmonary tuberculosis. A smear was made from each specimen, stained by the Ziehl-Neelsen method and examined microscopically for 5 minutes with the 1/7 inch fluorite oil-immersion lens. If acid-fast bacilli were observed the specimen was rejected; if acid-fast bacilli were not seen the specimen was divided into 2 equal parts, one for treatment by the proposed method and the other for treatment by the standard sodium hydroxide method. When dividing the specimen, care was taken to ensure that, as far as possible, all purulent parts of the sputum were distributed equally between the 2 portions. Arrangements were then made for the examination of 2 further specimens from patients who yielded specimens which were negative by microscopy. This procedure was repeated with fresh groups of patients until 3 replicate specimens had been provided by each of 40 to 50 patients.

The value of replicate specimens. In experiment 12 of this thesis (p. 159 and tables 74 to 79) it was found that a difference was demonstrated in the efficiency with which 2 methods produced cultures of tubercle bacilli from a series of specimens, each from a different patient and negative by microscopy for acid-fast bacilli; a greater difference, however, was observed in a smaller group of microscopically negative specimens which consisted of 2 or more replicate specimens from a group of 40 patients. Replicate specimens were therefore adopted for the critical assessment of methods of isolating tubercle bacilli from sputum. In this way some compensation was made for the fact that the studies reported in this thesis were made with relatively small numbers of specimens; it must be emphasised that one worker with the part-time assistance of one technician cannot undertake investigations on the scale of that conducted with the resources of 12 laboratories of the Public Health Laboratory Service (see p. 13 of this thesis).

METHODS USED FOR INDIVIDUAL EXPERIMENTS

Because the general methods already described may differ in small but important details from one experiment to another, details of methods and experimental procedure are given for each experiment.

Experiment 1. (a) A comparison of the numbers of viable tubercle bacilli present in untreated sputum with the numbers which remained in the sputum after the application of standard methods of preparing sputum for culture.

(b) The recovery of tubercle bacilli from swabs inoculated with dilutions of sputum.

(c) The numbers of viable tubercle bacilli discarded with the supernatant fluid after centrifugation.

This study was made with 7 specimens of sputum in all of which numerous acid-fast bacilli were demonstrated by microscopy.

Preparation of sputum for viable counts.

(a) Standard methods and control counts. Five portions, each of 2 ml. volume, were taken from each of the 7 specimens. A separate portion of each specimen was treated by one of each of the 4 following standard methods: the sodium hydroxide method, the sulphuric acid method, the acid-iron-peroxide method and the trisodium phosphate method. These methods were applied as described on pages 74 to 78 of this thesis as far as the first washing and centrifugation. The deposits obtained at this point by each method were resuspended in 2 ml. of Proskauer and Beck medium and from the suspensions dilutions

were made for viable counting.

The buffering action of the Proskauer and Beck medium used as a diluent in the viable counting method made it unnecessary to neutralise or wash the sediments. The fifth portion of each specimen served as an untreated control; from it were prepared 2 sets of dilutions for viable counts, one in Proskauer and Beck medium and the other in Proskauer and Beck medium containing 100 units of penicillin per ml. This procedure was adopted in order to obtain, if possible, counts of the numbers of viable tubercle bacilli from sputum which, apart from dilution, had not been treated in any way to suppress contaminants and in order to compare these counts with those obtained from untreated sputum diluted in Proskauer and Beck medium containing penicillin. In fact, the incorporation of penicillin in the diluting fluid did not seriously affect the numbers of tubercle bacilli in the 2 specimens in which a figure was obtained from untreated sputum prepared for counting without penicillin(see table 55).

(b) Viable counts from swabs. The dilutions of sputum in Proskauer and Beck medium without penicillin were used to transfer material to cotton wool swabs in order to establish the number of organisms which could be recovered from cotton wool swabs. Six of the 7 sputa were investigated in this way. For each sputum, three cotton wool swabs were charged

with material from each of the 4 weakest dilutions of sputum; the volume of material transferred to each swab was 0.02 ml. The swabs were then immersed in 2.5 per cent. oxalic acid; after 20 minutes at room temperature one slope of Lowenstein-Jensen medium was inoculated from each swab.

(c) Counts from supernatant fluid after centrifugation. In 2 of the 7 specimens viable counts were made from the supernatant fluids obtained after centrifuging the diluted homogenates prepared by each method. In order to compare the numbers of organisms isolated from the dilute supernatant, the volume of this fluid was measured and the difference between this volume and the original volume (2 ml.) of sputum treated was allowed for in making the dilutions for setting up the viable counts.

Experiment 2. A comparison of the numbers of viable tubercle bacilli in portions of specimens of sputum (a) untreated (b) homogenised with pancreatin (c) homogenised and decontaminated with sodium hydroxide and (d) homogenised and decontaminated with sulphuric acid.

Three specimens of sputum were used for this investigation. Each specimen was divided into 4 equal portions: the first portion was thoroughly mixed with an equal volume of sterile distilled water and dilutions were prepared for viable counting without further homogenisation; the second portion was mixed with an equal volume of 1 per cent. pancreatin solution and after an incubation period of 1 hour at 37°C dilutions were prepared from the homogenate for viable counting; the third portion was homogenised and decontaminated with an equal volume of 4 per cent. sodium hydroxide (see p. 74 of this thesis) and the fourth portion was homogenised and decontaminated with an equal volume of 3 per cent. (V/V) sulphuric acid and diluted for counting (see p. 75 of this thesis).

Diluting fluid. The untreated sputum and the sputum homogenised with pancreatin were diluted in Proskauer and Beck medium containing 1/250,000 proflavine sulphate. This concentration of proflavine was shown in experiment 6 (see p.146 of this thesis, table 64) to have no inhibiting effect on the growth of the H37Rv strain of tubercle bacilli; it was

used in the present experiment to prevent the growth of contaminants in counts from sputum which had not been treated with a decontaminating agent.

The homogenates prepared with sodium hydroxide or with sulphuric acid were diluted for counting in Proskauer and Beck medium without proflavine. Neutralisation of these homogenates was unnecessary because the buffering action of the diluent produces a neutral pH in the dilutions beyond 1:10.

Experiment 3. The effect of neutralisation on the numbers of viable organisms in homogenates of sputum prepared with acid or alkali.

Two groups of 3 specimens were examined; all specimens contained numerous acid-fast bacilli.

First group: Each specimen was divided into 2 equal portions; one portion was homogenised and decontaminated with an equal volume of 4 per cent. sodium hydroxide and the other with an equal volume of 3 per cent. (V/V) sulphuric acid. The sodium hydroxide homogenate was divided into 3 equal portions; using 3 per cent. (V/V) sulphuric acid, one portion was neutralised exactly, one portion received an excess of 0.1 ml. of acid after exact neutralisation and one portion was not neutralised. A similar procedure was used for the sulphuric acid homogenate and 4 per cent. sodium hydroxide was used as the neutralising agent. The volumes of all the portions of homogenised sputum were then made equal by adding the appropriate volume of sterile distilled water to each one. The number of viable tubercle bacilli in each portion was determined in the usual way.

Second group: The second group of specimens was treated in exactly the same way as the first except that 3 per cent.

(V/V) hydrochloric acid was substituted for sulphuric acid for homogenisation and 10 per cent. (V/V) hydrochloric acid was substituted for sulphuric acid in the neutralisation of alkaline homogenates.

Experiment 4. The ability of centrifugation to recover tubercle bacilli from sputum homogenised by the sodium hydroxide method.

A specimen of sputum containing numerous acid-fast bacilli was homogenised with an equal volume of 4 per cent. sodium hydroxide. Four portions, each of 4 ml. volume, were taken from the homogenate and treated as follows.

First portion. The volume of this portion was made up to 20 ml. with sterile distilled water. A viable count was then made from the diluted homogenate. This count gave the number of viable organisms present in the homogenate.

Second portion. The volume of this portion was made up to 20 ml. with sterile distilled water and centrifuged for 20 minutes. The supernatant was decanted into a fresh sterile container and a count was made of the number of viable organisms in the supernatant; this count gave the number of viable organisms discarded with the supernatant fluid after centrifuging homogenate which had not been neutralised. The sediment was resuspended in 20 ml. of sterile distilled water; 0.5 ml. of the resuspended sediment was removed for viable counting - this count gave the number of organisms recovered after diluting and centrifuging the

original homogenate. The remaining 19.5 ml. of resuspended sediment was centrifuged a second time and the deposit was suspended once more in 19.5 ml. of sterile distilled water; a count of the number of viable organisms in this suspension gave the numbers of viable organisms recovered after the two procedures used in the standard sodium hydroxide method (1) dilution and centrifugation of the original homogenate and (2) resuspension and centrifugation of the deposit obtained by the first centrifugation.

Third portion. This portion of homogenate was neutralised with 3 per cent, (V/V) sulphuric acid and the volume of the neutralised homogenate was made up to 20 ml. with sterile distilled water. A viable count of this neutralised and diluted homogenate gave the number of viable tubercle bacilli present after neutralisation.

Fourth portion. This portion was neutralised and diluted to a volume of 20 ml. as described above for the third portion. The neutralised and diluted homogenate was then centrifuged for 20 minutes. The supernatant was decanted into a fresh container and a viable count was made from the decanted fluid; this count gave the numbers of organisms present in the supernatant fluid after centrifuging neutralised homogenate.

The sediment was resuspended in 20 ml. of sterile distilled water and a viable count was made; this count gave the numbers of viable tubercle bacilli recovered by centrifuging the neutralised homogenate.

Experiment 5. The choice of a standard method of isolating tubercle bacilli from sputum.

The sodium hydroxide method (see p. 74 of this thesis) was compared with the sulphuric acid method (see p. 75 of this thesis).

Comparison of the efficiency of the two methods. The procedure described on p. 82 of this thesis was adopted for this experiment; 3 replicate specimens from each of 40 patients - a total of 120 specimens - were examined.

Experiment 6. The action of selective bacteriostatic agents on tubercle bacilli.

Media containing bacteriostatic agents. The basal medium was that of Proskauer and Beck with the addition of 10 per cent. sterile horse serum; it was dispensed in 1.8 ml. volumes in bijou bottles. A series of stock solutions in Proskauer and Beck medium was prepared for each bacteriostatic agent and media containing the agents were prepared by adding 0.2 ml. of stock solution to 1.8 ml. of the basal medium. For example, a series of stock solutions of ethyl violet was made with the following concentrations of dye: 1:1.000; 1:2,500; 1:5,000; 1:10,000; 1:25,000; 1:50,000; the addition of 0.2 ml. of each of these concentrations of dye to the basal medium produced a series of test media with the following concentrations of dye: 1:10,000; 1:25,000; 1:50,000; 1:100,000; 1:250,000; 1:500,000.

The following additional series of test media were made in the same way.

Agent	Range of concentrations
Proflavine	1:10,000, 1:25,000, 1:50,000, 1:100,000, 1:250,000, 1:500,000.
Malachite green	As above
Potassium tellurite	" "
Sodium oxide	" "
Silver nitrate	" "
Hibitane	" "
Oxalic acid	1:200, 1:2,000, 1:4,000, 1:5,000

Control medium: The control medium in this experiment consisted of 1.8 ml. of basal medium plus 0.2 ml. of Proskauer and Beck medium.

Inoculum: The H37Rv strain was used for these experiments. The inoculum consisted of a portion of a 10-day-old surface culture of this strain in Proskauer and Beck medium containing 10 per cent. of horse serum; the size of the inoculum was approximately 1 millimetre in diameter.

Incubation and recording of results. The inoculated media were incubated at 37°C. The results were recorded as follows.

Complete inhibition. "Complete inhibition" was recorded when the inoculum increased in size during an incubation period (usually 10 to 14 days) in which the surface of the control medium became completely covered with growth.

Partial inhibition. "Partial inhibition" was recorded when the inoculum increased in size but failed to cover the whole surface of the medium during a period in which the surface of the control medium became completely covered.

No inhibition. "No inhibition" was recorded when the inoculum covered the whole surface of the medium in a time not exceeding that in which the whole surface of the control medium became completely covered.

Experiment 7. The isolation of tubercle bacilli from sputum by means of combinations of pancreatin with bacteriostatic agents.

Pancreatin. Pancreatin (B.D.H.) was made up in a 2 per cent. solution in phosphate buffer at pH 7.6. The solution was left at 4°C and fresh solutions were made every 2 days.

Combinations of pancreatin with bacteriostatic agents. The following mixtures were made for homogenising and decontaminating sputum in order to isolate tubercle bacilli; the stated concentrations of pancreatin and bacteriostatic agent are those present in the mixture.

a) Pancreatin 1 per cent., malachite green 0.1 per cent.
b) Pancreatin 1 per cent., ethyl violet 0.1 per cent.
c) Pancreatin 1 per cent., proflavine 0.1 per cent.
d) Pancreatin 1 per cent., Hibitane 1 per cent.
e) Pancreatin 1 per cent., Desogen 1 per cent.
f) Pancreatin 1 per cent., silver nitrate 0.1 per cent.
g) Pancreatin 1 per cent., mercuric chloride 0.1 per cent.

Preparation of sputum for culture. Sputum was mixed with an equal volume of the mixture of pancreatin and bacteriostatic agent and incubated for 30 minutes at 37°C. The homogenate was then gently mixed by repeated aspiration into and discharge from a fine pasteur pipette fitted with a rubber teat. This is a potentially dangerous procedure and care was taken to avoid violent movement, splashing or bubbling of the homogenate. Two Löwenstein-Jensen slopes were inoculated from the homogenate; one slope received 3 drops of homogenate from a pasteur pipette, the other received 1 loopful of homogenate spread evenly over the surface of the medium.

Method of assessing the efficiency of the mixtures. Specimens of sputum containing large numbers of tubercle bacilli were used in order to indicate whether any of the mixtures of pancreatin with bacteriostatic agent deserved a more critical examination (see p. 81 of this thesis). The numbers of specimens used to test each method were as follows.

Method	Number of specimens
Pancreatin-malachite green	10
Pancreatin-ethyl violet	5
Pancreatin-proflavine	5
Pancreatin-hibitane	16
Pancreatin-desogen	6
Pancreatin-silver nitrate	4
Pancreatin-mercuric chloride	4

Experiment 8. The isolation of tubercle bacilli from sputum with combinations of different concentrations of trisodium phosphate with bacteriostatic agents.

The following mixtures of trisodium phosphate and bacteriostatic agents were used for preparing sputum for culture.

Trisodium phosphate with proflavine. Solutions of 10 per cent., 5 per cent. and 1 per cent. anhydrous trisodium phosphate were prepared, each containing 0.01 per cent. of of proflavine.

Trisodium phosphate with hibitane. Solutions of 10 per cent., 5 per cent. and 1 per cent. anhydrous trisodium phosphate were prepared, each containing 0.01 per cent. of hibitane.

Control solutions. Solutions of 10 per cent., 5 per cent. and 1 per cent. anhydrous trisodium phosphate were prepared without the addition of bacteriostatic agents.

Preparation of sputum for culture. Specimens of sputum were divided into 9 equal portions. Each portion was mixed with an equal volume of one of the above trisodium phosphate solutions. After an incubation period of 24 hours at 37°C the mixtures were neutralised with 5 per cent. (V/V) sulphuric

acid. A slope of Löwenstein-Jensen medium was inoculated from each homogenate; the inoculum consisted of 1 drop from a pasteur pipette.

Method of assessing the efficiency of the mixtures. As in experiment 7 only a preliminary trial was carried out with the mixtures described above (see p. 81 of this thesis). Four specimens were examined; each contained numerous tubercle bacilli and was examined as described above.

Experiment 9. The use of Teepol in the isolation of tubercle bacilli from sputum.

Browning's method.

Sputum was mixed with an equal volume of Teepol. After 4 hours 2 Löwenstein-Jensen slopes were inoculated from the homogenate; one slope received 1 loopful of homogenate evenly distributed over the surface of the medium, the other received 4 drops of homogenate from a pasteur pipette.

Method of assessing the efficiency of Browning's method. The method was tested with 5 specimens of sputum known to contain acid-fast bacilli. Only a preliminary trial was made (see p. 81 of this thesis).

The sodium hydroxide teepol method.

A solution was prepared containing 1 per cent. sodium hydroxide and 25 per cent. Teepol. This was used for homogenising and decontaminating sputum specimens in the manner described for 4 per cent. sodium hydroxide (see p. 74 of this thesis).

Preparation of sputum for inoculation. Although the solution used for homogenisation and decontaminating is that described by Tison, the details of its use differ in several important respects from those prescribed by Tison. First, the neutralisation procedure is omitted. Second, the time of centri-

fugation is 20 minutes instead of 1 hour. Third, the homogenised material was washed only once instead of several times. No doubt the simplified method described above may yield less satisfactory results than those claimed by Tison for his original method (see p. 31 and tables 28 and 29 of this thesis). The aim of the present experiment is to find out whether a simplification of Tison's method is more efficient than the sodium hydroxide method; the efficiency of Tison's own procedure was not investigated because it in unsuitable for routine laboratory practice; the centrifugation time alone makes it impracticable for large numbers of routine specimens.

Method of assessing the efficiency of the sodium hydroxide-teepol method. The procedure described on p. 82 of this thesis was adopted; 3 replicate specimens were examined from each of 15 patients - a total of 45 specimens. The efficiency of sodium hydroxide-teepol method was compared with that of the standard sodium hydroxide method. The number of patients examined was small because the experiment was terminated as soon as it became clear that the modified sodium hydroxide-teepol method was no more efficient than the sodium hydroxide method.

Experiment 10. The importance of centrifugation in the sodium hydroxide method.

Preparation of sputum for culture. Each specimen of sputum was homogenised and decontaminated with an equal volume of 4 per cent. sodium hydroxide (see the sodium hydroxide method, step (a) p. 74 of this thesis). One - tenth of the homogenate was transferred to a separate container, and neutralised with 3 per cent. (V/V) of sulphuric acid; all of the neutralised homogenate was inoculated on 2 slopes of Löwenstein-Jensen medium by means of a sterile pasteur pipette. The remaining nine-tenths of ths sputum was prepared for culture by centrifugation and washing as described in steps (b) and (c) of the sodium hydroxide method on p. 75 of this thesis.

Method of assessing the efficiency of the sodium hydroxide method in which centrifugation was omitted. A series of 74 specimens of sputum, was examined. Each specimen was obtained from a different patient and was negative by microscopy for acid-fast bacilli. The homogenised specimens were treated as described above and the results of culture without centrifugation were compared with those in which washing and centrifugation were carried out.

Experiment 11. Fluid media for the isolation of tubercle bacilli from sputum.

In this experiment the E.V. medium and the P.F. medium described on page 74 of this thesis were used for the isolation of tubercle bacilli from sputum in comparison with the standard sodium hydroxide method.

Trial of the E.V. medium.

Sputum specimens. Thirty-two specimens of sputum were cultured in E.V. medium and by the sodium hydroxide method. In all of the specimens direct microscopy failed to detect acid-fast bacilli. After concentration by the sodium hydroxide method 7 of the 32 specimens were shown by microscopy to contain a few acid-fast bacilli; the remaining 25 specimens were negative for acid-fast bacilli even after concentration.

Preparation of sputum for culture. Each specimen was carefully divided into 2 equal portions (see p. 82 of this thesis).

One portion was transferred to 30 ml. of E.V. medium contained in a flat screw-capped bottle of 100 ml. capacity. The inoculated bottle was incubated in a horizontal position at 37°C in order to provide the maximum surface area for the free passage of oxygen into the medium. At weekly intervals for 8 weeks smears were made from the

sediment of the medium, stained by the Ziehl-Neelsen method and examined microscopically with the 2/3 inch objective for the presence of microcolonies of tubercle bacilli.

The second portion was cultured by the standard sodium hydroxide method as described on p. 74 of this thesis.

Trial of the P.F. medium.

Sputum specimens. Twenty-seven specimens were cultured in the P.F. medium and by the sodium hydroxide method. In 3 of the specimens acid-fast bacilli were detected by direct microscopy; in a further 5 of the specimens acid-fast bacilli were detected by microscopy only after concentration by the sodium hydroxide method; in the remaining 19 specimens acid-fast bacilli were not detected even after concentration.

Preparation of sputum for culture. Each specimen was carefully divided into 2 equal portions (see p. 82 of this thesis).

One portion was homogenised and decontaminated with an equal volume of 1 per cent. sodium hydroxide by incubating at 37°C for 20 minutes. The homogenate was then transferred to a flat 100 ml. screw-capped bottle containing 20 ml. of P.F. medium. Neutralisation of the homogenate was unnecessary because the buffering action of the P.F. medium was sufficient to maintain a pH of 6.8 to 7.4

after inoculation with the sodium hydroxide homogenate. The inoculated medium was incubated in a horizontal position for 8 weeks and examined for microcolonies of tubercle bacilli as described for the E.V. medium on p.104 of this thesis.

The second portion was cultured by the sodium hydroxide method as described on p. 74 of this thesis.

Experiment 12. The evaluation of Nassau's swab method of isolating tubercle bacilli from sputum.

Preparation of sputum for culture by Nassau's swab method. Two sterile swabs mounted on orange sticks were moistened in distilled water. They were then immersed and rotated in the specimen of sputum so that some sputum adhered to them. Each swab was placed in a sterile tube (5" x $\frac{5}{4}$") and the tube was half-filled with 5 per cent. oxalic acid. After 25 minutes at room temperature each swab was transferred to a second tube containing 5 per cent. sodium citrate to neutralise the acid. After 10 minutes a separate slope of Löwenstein-Jensen medium was inoculated from each swab. The swab was pressed and rotated on the medium so that most of the sputum was transferred to the medium. The swabs were then discarded into lysol. Inoculated slopes were incubated for 8 weeks at 37°C and examined at weekly intervals.

Method of assessing the efficiency of the swab method. Four groups of specimens were examined.

The first group consisted of 96 specimens in which acid-fast bacilli were demonstrated by microscopy. These specimens were cultured only by the swab method in order to establish whether or not the method deserved a more

critical examination (see p. 81 of this thesis).

There were 30 specimens in the second group; they were examined by the swab method and by the standard sodium hydroxide method. In the specimens in this group, acid-fast bacilli were demonstrated by microscopy but only after concentration by the sodium hydroxide method.

The third group consisted of 130 specimens in which acid-fast bacilli were not seen even after concentration by the sodium hydroxide method. Each specimen was obtained from a different patient and was examined by the swab method and by the sodium hydroxide method (see p. 74 of this thesis).

The fourth group of specimens also consisted of specimens in which acid-fast bacilli were not seen by microscopy. There were 104 specimens in this group but they were obtained from only 41 patients, each patient contributing 2 or more replicate specimens. Each specimen was cultured by the swab method and by the sodium hydroxide method. The critical procedure described on p. 82 of this thesis was not adopted for this experiment because, although this experiment appears as the twelfth experiment it was carried out before the value of the examination of replicate specimens was appreciated; it was in fact, the experiment which drew my attention to the value of replicate specimens for the critical assessment of methods

of isolating tubercle bacilli from sputum in circumstances in which it was not possible to examine series of specimens on the scale adopted in the investigation made by the Public Health Laboratory Service (see p. 13 of this thesis).

Experiment 13. Comparison of sputum culture and laryngeal swab culture for the isolation of tubercle bacilli from patients suffering from pulmonary tuberculosis.

Laryngeal swabs. The swabs consisted of a generous wrapping of nylon wool on the roughened end of 9 inches of brass wire. Nylon wool was used instead of cotton wool because Thomas (1956) showed that tubercle bacilli were more easily recovered from nylon wool than from cotton wool. The swabs were assembled in stoppered glass tubes as illustrated in fig. 3 and the assembled swabs were sterilised by autoclaving at 15 lbs. pressure for 20 minutes.

Laryngeal swab specimens. As the wire was withdrawn from the sterile tube it was bent approximately 2½ inches from the swab end to form an angle of approximately 100 degrees with the main part of the wire. The nylon swab was then moistened in sterile distilled water. The patient was instructed to pull his tongue well forward with a gauze swab and the nylon swab was passed into the pharynx. Then, without the aid of a laryngeal mirror, the swab was rapidly manoeuvred towards the larynx. At this point

the patient usually coughed; if he did not cough he was instructed to do so. The swab was then withdrawn from the patient and as it was replaced in the sterile tube the wire was straightened against the side of the glass. The operator was protected from infection by wearing a surgical gown, cap, mask and a transperent face visor made from old X-ray film.

Preparation of laryngeal swab specimens for culture. The swabs were decontaminated with oxalic acid as described on p.107 of this thesis for Nassau's swab method.

Assessment of the efficiency of the laryngeal swab method. A procedure similar to the critical procedure described on p.82 of this thesis was adopted. A group of 46 patients was obtained whose sputum specimens were negative for acid-fast bacilli by direct examination. Each patient contributed a specimen of sputum on each of 3 consecutive days. When each specimen of sputum was collected two laryngeal swabs were taken from the patient. The specimens of sputum were cultured by the sodium hydroxide method (see p. 74 of this thesis). In this way, for each patient, the results of 3 replicate cultures by laryngeal swab, each involving the examination of 2 specimens, were compared with the results of 3 sputum cultures made by the sodium hydroxide method;

both kinds of examination were made from specimens collected at approximately the same time.

Experiment 14. Concentration of tubercle bacilli from homogenates of sputum by sedimentation with barium sulphate.

Barium sulphate suspension. A 10 per cent. suspension of barium sulphate B.P. in distilled water was used.

Apparatus. Sedimentation tubes were made of stout borosilicate glass tubes (fig. 4). The tubes were 7" long; the top 5 inches had an internal diameter of 3/4" and the bottom 2 inches consisted of a tapered portion 1 inch long leading to a straight terminal portion 1 inch long with a diameter of $\frac{1}{4}$ inch. A one-holed stopper carrying an 8-inch glass rod was fitted to the top portion of the tube and a 2-inch length of rubber tube was fitted to the narrow bottom part of the tube. The rubber tube contained a glass bead; the diameter of the bead was slightly larger than that of the rubber tube. The bead acted as a stop-cock; when the rubber tube was pinched firmly at the level of the glass bead, distortion of the rubber tube permitted free flow of fluid. The glass bead was preferred to a spring clip because it facilitated the preparation and storage of a large number of tubes. When the tubes were assembled for sterilisation the terminal rubber portions were wrapped in lead foil (fig. 5); the assembled tubes were sterilised by autoclaving at a pressure of 15 lbs.

per square inch for 20 minutes.

Preparation of sputum for culture by barium sulphate sedimentation.

(a) Homogenisation and decontamination with sodium hydroxide. Sterile sedimentation tubes were set up in a rack (Fig. 6), two for each specimen. The first tube was empty and the second contained approximately 20 ml. of phosphate buffer at pH 7 (fig. 6, tubes 1 and 2). Specimens of sputum were homogenised and decontaminated with an equal volume of 4 per cent. sodium hydroxide as described in step (a) of the sodium hydroxide method on p. 74 of this thesis. The volume of the homogenate was made up to 20 ml. with sterile distilled water and 1 ml. of barium sulphate suspension was added. The mixture was then inverted twice and poured carefully into the empty sedimentation tube. After 10 minutes the appearance of the two tubes was that shown in fig. 6, tubes 3 and 4; the lead foil was then removed from the rubber portion of the tube containing the homogenised specimen (fig. 6, tube 3); by compressing the rubber tube at the level of the glass bead the sediment was run into the second sedimentation tube containing sterile phosphate buffer at pH 7 (fig. 6, tube 4). The sediment was dispersed in the buffer by means of the glass rod. After 10 minutes the appearance of the two tubes is that shown in

fig. 6, tubes 5 and 6. The lead foil was then removed from the end of the second tube and the sediment was released into 2 slopes of Löwenstein-Jensen medium. After use, the sedimentation tubes were discarded into a watertight metal box and sterilised by autoclaving at a pressure of 10 lb. per square inch for 20 minutes before washing.

(b) Homogenisation with a mixture of trisodium phosphate and benzalkonium chloride. Patterson et al. (1956) described a method of isolating tubercle bacilli from sputum by homogenisation and decontamination with a mixture of trisodium phosphate and benzalkonium chloride. The method differs from that described on p. 74 of this thesis for the standard sodium hydroxide method in only 3 respects. First, the agent used for homogenisation and decontamination of the sputum is a solution containing 10 per cent. trisodium phosphate (anhydrous salt) and 0.1 per cent. benzalkonium chloride; second, the time of incubation of the mixture of sputum and decontaminating agent is only 15 minutes; and third, the process of decontamination and homogenisation is carried out at room temperature. Because Patterson et al. claim that their method is more efficient than either the sodium hydroxide method or the trisodium phosphate method it was decided to assess the efficiency of a modification of the method of Patterson et al. in which sedimentation with barium sulphate was substituted for centrifugation. Details of the preparation of sputum for

culture by the modified trisodium phosphate - benzalkonium chloride method differ from those of the sodium hydroxide - barium sulphate method (p. 114 above) only in the following respects: 1) the sputum was homogenised and decontaminated with trisodium phosphate-benzalkonium chloride mixture instead of sodium hydroxide and 2) homogenisation and decontamination was limited to 15 minutes at room temperature.

Assessment of the efficiency of the sodium hydroxide-barium sulphate method. The critical procedure described on p. 82 of this thesis was applied. Only a preliminary investigation was made involving the examination of 3 replicate specimens from each of 16 patients - a total of 48 specimens - by the sodium hydroxide-barium sulphate method and by the standard sodium hydroxide method. The results of this investigation (p. 169 and table 83 of this thesis) indicated that sedimentation with barium sulphate might be a good alternative to centrifugation and therefore this method of concentrating tubercle bacilli from homogenates of sputum was examined more critically. For the reason given above (p. 115 of this thesis) homogenates prepared by the method of Patterson et al. were used for the critical examination of the efficiency of the barium sulphate method of concentrating tubercle bacilli.

Assessment of the efficiency of the trisodium phosphate - benzalkonium chloride-barium sulphate method. The efficiency of the trisodium phosphate - benzalkonium chloride - barium sulphate method was compared with that of the standard sodium

hydroxide method. The critical procedure described on p. 82 of this thesis was adopted for this comparison. Three replicate specimens from 52 patients - a total of 156 specimens - were examined by both methods.

INDIVIDUAL EXPERIMENTS

RESULTS AND ANALYSIS

A. Quantitative studies based on counts of viable tubercle bacilli.

The possible advantages of applying a viable counting procedure to the investigation of methods of isolating tubercle bacilli from sputum are as follows. First, specimens of sputum containing large numbers of tubercle bacilli are readily obtainable. Second, the efficiency of methods may be compared in terms of the numbers of tubercle bacilli which survive the application of these methods. Third, the details of the methods may readily be examined: for example, the efficiency with which tubercle bacilli may be recovered from a homogenate by centrifugation and the lethal effect of neutralisation procedures. Fourth, it may be possible to assess the lethal effects of methods not only in relation to each other as in (2) above but in terms of the numbers of tubercle bacilli present in the untreated specimen; in other words it may be possible to assess the methods in absolute terms and thus obtain information which will decide whether or not there is any hope of introducing new methods which, because they kill significantly fewer tubercle bacilli, will be superior to existing methods. This fourth possibility may be realised under two sets of circumstances (1) when

the tubercle bacilli outnumber the other organisms in the sputum and can therefore be enumerated without interference by contamination in the weaker dilutions of sputum and (2) if a suitable bacteriostatic agent is incorporated in the diluting fluid.

Experiment 1.

(a) A comparison of the numbers of viable tubercle bacilli present in untreated sputum with the numbers remaining in the same sputum after the application of standard methods of preparing sputum for culture.

(b) The recovery of tubercle bacilli from swabs inoculated with dilutions of sputum.

(c) The numbers of viable tubercle bacilli discarded with the supernatant fluid after centrifugation.

Seven specimens of sputum were studied by means of the viable counting procedure described on p. 78 of this thesis. The following counts were made:-

(1) The numbers of tubercle bacilli present in untreated portions of sputum. Two sets of counts were made for each of the 7 specimens, the first using Proskauer and Beck medium as the diluent and the second using the same diluent containing 100 units per ml. of penicillin in order to suppress contaminants.

(2) The numbers of tubercle bacilli in portions of sputum treated with sodium hydroxide, trisodium phosphate, sulphuric acid or acid-iron-peroxide (see pages 74 to 78 of this thesis).

These counts were made for each of the 7 specimens of sputum and were made from suspensions of deposits obtained by centrifuging sputum homogenates.

(3) The numbers of tubercle bacilli which could be recovered from cotton wool swabs charged with 0.02 ml. (the volume chosen for inoculating the media used for viable counts, see p. 79 of this thesis) of diluted sputum and treated with oxalic acid. The 4 highest dilutions of untreated sputum prepared for the counts in (1) above were used for this purpose; the numbers of tubercle bacilli recovered from the swabs may therefore be compared directly with those obtained in (1) and (2) above. This investigation was made with all of the 7 specimens of sputum except number 6.

(4) Counts were made of the numbers of tubercle bacilli present in the supernatant fluid obtained by centrifuging the homogenates prepared by the sodium hydroxide, trisodium phosphate, sulphuric acid and acid-iron-peroxide methods from portions of specimen number 5 and specimen 6 (see p. 86 of this thesis).

The results of the first 3 sets of counts are presented in table 55; the results of the fourth set are presented in table 56. The following points deserve comment:-

Numbers of tubercle bacilli present in untreated portions of sputum. Successful counts without the aid of penicillin of the numbers of tubercle bacilli present in untreated portions of sputum were obtained only with 2 specimens, numbers

4 and 6. In these two specimens the count obtained with the aid of penicillin was approximately equal to that obtained without penicillin. It is reasonable, therefore, to regard the counts obtained only with the aid of penicillin in 4 of the specimens (numbers 1, 2, 5 and 7) as a true estimate of the numbers of viable tubercle bacilli present in untreated sputum. A control figure is therefore available for the numbers of tubercle bacilli in the untreated portions of 6 of the 7 specimens, numbers 1, 2, 4, 5, 6 and 7; because both sets of counts from untreated sputum were destroyed by contamination there is no control figure for sputum number 3.

Comparison of the numbers of tubercle bacilli present in untreated portions of sputum with the numbers in portions treated with decontaminating agents. The control count is higher than the counts obtained from sputum treated with decontaminating agents in 4 specimens, numbers 2, 4, 5 and 6; it is not significantly lower than the counts obtained from sputum treated with decontaminating agents in the 2 other specimens for which control counts are available, numbers 1 and 7. Although the orders of efficiency of the sodium hydroxide, trisodium phosphate, sulphuric acid and acid-iron-peroxide methods are not constant from specimen to specimen, the sulphuric acid method yields the highest count apart from the control in 5 of the specimens, numbers

2, 3, 4, 6 and 7; moreover, in 4 specimens, numbers 2, 3, 5 and 7 the sulphuric acid method and the acid-iron-peroxide method both yield higher counts than either of the alkali methods. It must be emphasised that the degree of difference between methods is irregular; it may be almost negligible as in specimens 1 and 5, apparently significant as in specimens 2, 4, 6 and 7, or of doubtful significance as in specimen 3.

The lethal effect of decontaminating agents. The results in table 55 show that the lethal effect of all of the methods is irregular. In specimen 1, the lethal effect of all of the methods is so small as to suggest that any of the 4 methods of decontaminating sputum should always detect viable tubercle bacilli if any are present in the specimen. The range of the lethal effect is least with the sulphuric acid method, from no lethal effect (specimens 1 and 7) to a survival equal to less than one-third of the control figure (specimen 5). The ranges for the other methods are as follows: the sodium hydroxide method - none (specimen 1) to a survival equal to one-eighth of the control figure (specimen 6); the trisodium phosphate method - none (specimen 1) to a survival equal to less than one-tenth of the control (specimen 6); the acid-iron-peroxide method - from a survival equal to four-fifths of the control figure (specimen 1) to a survival of less than one-fourteenth of the control figure (specimen 6).

The recovery of tubercle bacilli from cotton-wool swabs. The numbers of tubercle bacilli recovered from cotton-wool swabs is very low in all the specimens in which this point was investigated. The greatest number of organisms isolated was less than one half of the control figure (specimen 7); in all other specimens the proportion of tubercle bacilli recovered from swabs was much less than this. It must be emphasised, however, that the swabs were charged with very dilute sputum which readily soaked into the cotton-wool; the recovery figures in table 55 do not necessarily give a reliable indication of the possible recovery of tubercle bacilli from swabs charged with sputum or with material from the larynx.

Tubercle bacilli discarded with the supernatant fluid after centrifugation. In the preparation of the treated portions of sputum for viable counting, homogenised sputum was centrifuged and the sediment retained for counting (see p. 84 of this thesis). Clearly, if large numbers of tubercle bacilli were to remain in the discarded supernatant fluid, the figures obtained for the numbers of tubercle bacilli which survived treatment with decontaminating agents would be inaccurate. Table 56 compares the numbers of tubercle bacilli present in the supernatant fluids with the numbers present in sediment after centrifuging homogenates prepared

by the different methods from specimens 5 and 6. Although tubercle bacilli are present in the supernatant fluid of all centrifuged homogenates the numbers are never more than one-fifth of the number present in the deposit (specimen 5, trisodium phosphate method). The differences in the lethal effects of the methods shown in table 55 cannot therefore be explained by the loss of a large proportion of tubercle bacilli from homogenates when the supernatant fluid is discarded after centrifugation.

One feature of these results must be emphasised - they do not indicate whether or not it is worth while to investigate other methods because the absolute lethal effect of each method differs from specimen to specimen.

Experiment 2

A comparison of the numbers of viable tubercle bacilli in portions of specimens of sputum (a) untreated, (b) homogenised with pancreatin, (c) homogenised and decontaminated with sodium hydroxide and (d) homogenised and decontaminated with sulphuric acid

In the previous experiment the following possibilities may have interfered with the accuracy of the results. First, the counts for untreated sputum may have been too low because the sputum was not homogenised. Second, the counts for alkaline and acid homogenates may have been upset by centrifugation; although there was only a small loss of tubercle bacilli in the discarded supernatant fluid after centrifugation, it is possible that the tubercle bacilli in the deposit were not resuspended homogeneously before counting because they might have adhered to the glass container or to one another. In order to clarify these points the following counts were made from 3 specimens of sputum:-

1) The numbers of tubercle bacilli present in untreated portions of sputum. In order to suppress contaminants, proflavine was incorporated in the diluting fluid (see p. 87 of this thesis).

2) The numbers of tubercle bacilli present in portions of sputum homogenised with pancreatin but not treated with strong acid or alkali. These counts were also made in diluting fluid containing proflavine.

3) The numbers of tubercle bacilli in portions of sputum treated with sodium hydroxide or sulphuric acid. These counts were made in diluting fluid without proflavine. The homogenates were diluted directly for counting without recovery of the tubercle bacilli by centrifugation; the alkali or acid in the homogenate was satisfactorily neutralised by the buffering action of the diluent.

The results are summarised in table 57.

Effect of homogenisation on untreated sputum. The counts from sputum homogenised with pancreatin are usually higher than those from sputum counted without homogenisation. Nevertheless, the differences in favour of counts from homogenised sputum were very small. It is reasonable, therefore, to assume that the counts for untreated sputum obtained in experiment 1 represent the numbers of tubercle bacilli present in untreated sputum.

Comparison of the lethal effects of sodium hydroxide and sulphuric acid. There is little difference in the lethal effects of sodium hydroxide and sulphuric acid on tubercle bacilli contained in sputum. A difference in favour of sodium

hydroxide was obtained with sputum No. 1 and the reverse with sputum No. 2 and sputum No. 3. On the other hand, untreated sputum or sputum homogenised with pancreatin consistently yielded a higher count than sputum homogenised with sodium hydroxide or sulphuric acid. These results are much more regular than those reported in experiment 1 (table 55) and suggest that centrifugation in experiment 1 did upset the comparison of lethal effects of the methods. However, the number of tubercle bacilli recovered from portions of sputum after treatment with sulphuric acid or with sodium hydroxide is more than half the number recovered from portions of the sputum which have not been exposed to these agents.

Experiment 3.

The effect of neutralisation on the numbers of viable organisms in homogenates of sputum prepared with acid or alkali.

If strong alkali or acid is used to decontaminate sputum in order to obtain a pure culture of tubercle bacilli, the homogenate must be freed from these substances before culture. This may be accomplished by dilution, washing or chemical neutralisation. Chemical neutralisation is most frequently used with the sodium hydroxide method. In Petroff's original method the whole homogenate is neutralised before centrifugation but present practice is to dilute and centrifuge the homogenate and neutralise the deposit before inoculating culture media. In the sulphuric acid method used by the Public Health Laboratory Service and in the oxalic acid method used by Corper and Uyei, the homogenate is diluted before centrifugation and the sediment obtained by centrifugation is used to inoculate culture media without neutralisation. Collins (see p. 19 of this thesis) suggests that this practice produces a low pH in the culture medium and reduces the chance of a positive result. If a chemical neutralisation procedure is adopted, the addition of a small excess of the neutralising agent will expose the tubercle bacilli in the specimen to the action of a second agent likely to diminish the numbers of

viable organisms. The aim of the experiment was therefore to determine the lethal effect of neutralisation procedures on tubercle bacilli in sputum.

Two groups of 3 specimens were examined. The first group of specimens was examined in the following way. Each specimen was divided into 2 equal portions; one portion was homogenised with sodium hydroxide and the other with sulphuric acid. Each homogenate was then divided into 3 equal portions; the first was not neutralised; the second was neutralised exactly; and the third received a small excess of the neutralising agent. From these portions of homogenised sputum the following counts were made for each specimen:-

1) The numbers of tubercle bacilli present in sputum homogenates prepared by the sodium hydroxide and sulphuric acid methods.

2) The numbers of tubercle bacilli present in neutralised sputum homogenates prepared by the sodium hydroxide method and the sulphuric acid method.

3) The numbers of tubercle bacilli present in "over-neutralised" sputum homogenates prepared by the sodium hydroxide method and the sulphuric acid method.

The second group of specimens was treated in a similar way except that hydrochloric acid was substituted for sulphuric

acid. From the homogenised sputum the following counts were made for each specimen:-

1) The numbers of tubercle bacilli present in sputum homogenates prepared by the sodium hydroxide method and the hydrochloric acid method.

2) The numbers of tubercle bacilli present in neutralised sputum homogenates prepared by the sodium hydroxide method and the hydrochloric acid method.

3) The numbers of tubercle bacilli present in over-neutralised sputum homogenates prepared by the sodium hydroxide method and by the hydrochloric acid method.

The results are presented in tables 58 and 59. They show that the exact neutralisation of the sodium hydroxide homogenates has no significant effect on tubercle bacilli but that the addition of a slight excess of acid during neutralisation may cause a marked drop in the number of viable tubercle bacilli (table 58 Sputum no. 1; table 59 sputum no. 5, sputum no. 6). On the other hand, with acid homogenates neither exact neutralisation nor the addition of an excess of alkali in the neutralisation procedure diminished the numbers of viable tubercle bacilli (table 5 sputum 1, 2, 3; table 6 sputum 4, 5, 6).

In addition, the results for homogenates which were not neutralised or which were neutralised exactly fail to show major differences between the lethal effects of alkali and acid. In table 58 the greatest difference is seen with

specimen 1 in which the numbers of tubercle bacilli surviving sodium hydroxide treatment is almost twice the number surviving sulphuric acid treatment. In table 59 the greatest difference is of the same order of magnitude but in this case, sputum no. 6, the difference is in favour of acid homogenisation. These results suggest that the neutralisation of homogenates prepared by the sodium hydroxide method may diminish considerably the numbers of viable tubercle bacilli if the end-point is exceeded whereas neutralisation of acid homogenates is unlikely to diminish the number of viable tubercle bacilli even if the end-point is exceeded. An important difference in the primary lethal action of acid and alkaline methods of homogenisation was not observed in this experiment.

Experiment 4.

The ability of centrifugation to recover tubercle bacilli from sputum homogenised by the sodium hydroxide method.

Most methods of isolating tubercle bacilli from sputum involve centrifugation. The primary aim of centrifugation is to concentrate the tubercle bacilli from a relatively large volume of sputum, 2 ml. or more into a small volume suitable for inoculation. It may also be used in order to wash the inoculum free of acid or alkali. The work of Klein et al. (see p. 49 of this thesis), Hata et al. (see p. 49 and table 48 of this thesis) and of Saxholm (see p. 49 and tables 50 to 54 of this thesis) casts doubt on the value of centrifugation for the collection of tubercle bacilli from a homogenate and if these doubts were substantiated it would be worth while to omit centrifugation from methods of isolating tubercle bacilli from sputum - a time-consuming and dangerous procedure. The present experiment was carried out to determine what proportion of tubercle bacilli could be collected by centrifugation from a homogenate prepared by the sodium hydroxide method. The following counts were made:-

1) The numbers of tubercle bacilli present in sputum

homogenised with sodium hydroxide but not neutralised.

2) The numbers of tubercle bacilli recovered in the deposit obtained by centrifuging homogenised sputum.

3) The numbers of tubercle bacilli in the supernatant fluid obtained by centrifuging homogenised sputum.

4) The numbers of tubercle bacilli recovered by centrifugation when the organisms recovered in (2) above were resuspended in distilled water.

These counts measure the effects of all the procedures used in the sodium hydroxide method described on p. 74 of this thesis.

In addition, the following counts were made from the same sputum homogenised by the sodium hydroxide method and neutralised before counting:-

1) The numbers of tubercle bacilli present in neutralised sodium hydroxide homogenate.

2) The numbers of tubercle bacilli recovered in the sediment obtained by centrifuging the neutralised homogenate.

3) The numbers of tubercle bacilli in the supernatant obtained by centrifuging the neutralised homogenate.

These additional counts measure the effects of all the procedures used in Petroff's original sodium hydroxide method described on p. 9 of this thesis.

The results are presented in table 60; they show

that centrifugation of a homogenate of sputum prepared by the sodium hydroxide method recovers most of the organisms of tubercle bacilli from the homogenate and that resuspension and centrifugation of the organisms recovered by the first centrifugation does not result in the loss of many tubercle bacilli. With a homogenate which has not been neutralised, although most of the organisms are collected in the sediment by centrifugation, the supernatant fluid remains rich in tubercle bacilli and contains more than one-tenth of the number originally present in the homogenate. On the other hand, centrifugation of the neutralised homogenate recovered most of the tubercle bacilli and the supernatant fluid did not retain sufficient tubercle bacilli to yield a count by the method adopted. Despite the complete clearing of the supernatant fewer tubercle bacilli were found in the deposit obtained by centrifugation of the neutralised homogenate. This could be explained by the fact that a precipitate is formed as a result of neutralisation; clumping of the tubercle bacilli in this precipitate would account for the slightly lower viable count after neutralisation. It is probable that the precipitate formed by neutralisation of the homogenate entangled the suspended tubercle bacilli and ensured complete removal of tubercle bacilli from the supernatant fluid. An alternative explanation is that suggested by the results of experiment 3 - that neutralisation had a lethal effect on the tubercle

bacilli.

General conclusions drawn from experiments 1 to 4.

1) The order of efficiency of methods of isolating tubercle bacilli from sputum is not constant in different specimens of sputum.

2) Comparison of the absolute lethal effects of different methods shows that, (a) on the whole, the sulphuric acid method is the least lethal to tubercle bacilli contained in sputum; (b) that the degree of lethal effect of this method and other methods differs over a wide range of values.

3) Relatively few tubercle bacilli may be recovered from swabs charged with very dilute suspensions of sputum.

4) In certain specimens of sputum, neutralisation of sodium hydroxide homogenates produces a marked drop in the number of viable tubercle bacilli contained in the homogenate; this effect was not observed in all the specimens examined. On the other hand, in all the specimens examined, neutralisation of acid homogenates did not affect the number of viable tubercle bacilli contained in the homogenates.

5) Centrifugation is an efficient method of recovering tubercle bacilli from an alkaline homogenate of sputum. If the homogenate is neutralised before centrifugation, the

supernatant fluid is cleared of tubercle bacilli; on the other hand, if the homogenate is not neutralised before centrifugation, the supernatant may contain more than one tenth of the numbers of tubercle bacilli originally present in the homogenate.

6) The information yielded by these studies is insufficient to decide the relative merits of the methods studied. Although the sulphuric acid method is shown to be superior, this conclusion is contrary to that of the study conducted by the Public Health Laboratory Service under conditions closely ressembling those which apply in routine laboratory practice. Experiments 1 to 4 were conducted under rather artificial conditions and with relatively few specimens. Clearly, the results obtained under the conditions of routine laboratory practice must carry more weight. It was decided, therefore, to use series of specimens of sputum for further comparisons of methods in this thesis.

B. Comparisons with a standard method of the numbers of positive results obtained by different methods in series of specimens of sputum.

Experiment 5.

The choice of a standard method for use in assessing the relative efficiency of recently described or original methods.

From the survey of the literature the sodium hydroxide method appears to be the most suitable for establishing the relative efficiency of a given method. There are two reasons for favouring the sodium hydroxide method: (1) it is significantly more efficient than the three other established methods of isolating tubercle bacilli in the study conducted by the Public Health Laboratory Service (see table 2 of this thesis) and (2) other studies with series of specimens of sputum have failed to demonstrate that any other method is significantly more efficient than the sodium hydroxide method. In the Public Health Laboratory Service trial, the sulphuric acid method was the second most efficient method. Because a neutralisation procedure had not been incorporated in the sulphuric acid method in the Public Health Laboratory Service trial it was decided to compare the sodium hydroxide method with a sulphuric acid method in which the concentrate produced

by centrifuging the homogenised sputum was neutralised with sodium citrate (see p. 75 of this thesis).

The two methods were compared in a series of replicate specimens of sputum obtained from 40 patients. Each patient contributed 3 specimens to the series and the total number of specimens examined was 120. Each specimen was negative for acid-fast bacilli by microscopy and was divided into 2 equal portions, one portion being allocated to each method.

The results are presented in two ways: (1) a comparison of the numbers of patients who yielded positive cultures and (2) a comparison of the numbers of specimens which yielded positive results.

Patients who yielded a positive culture. Table 61 shows the results obtained in the 3 replicate specimens contributed by each of the 11 patients who yielded a positive culture. The following points emerge:-

1) The sodium hydroxide method yielded a culture of tubercle bacilli from all of the 11 patients in at least 1 of the replicate specimens; on the other hand, the sulphuric acid method failed to detect tubercle bacilli in all 3 replicate specimens in 3 patients (2, 4 and 9).

2) When the specimens are divided into 3 separate series of specimens according to the chronological order in which each

specimen from each patient was examined, the difference between the two methods is small in any one series; the difference is in favour of sodium hydroxide method in 2 of the series and in favour of the sulphuric acid method in the remaining series.

3) The results are not greatly influenced by the loss of cultures by contamination. In most instances each method had the chance of yielding a positive or negative result on 2 slopes of Löwenstein-Jensen medium and in all instances each method had the chance of yielding a positive or negative result on at least 1 slope of medium.

Specimens which yielded positive cultures. Table 62 shows that the total number of positive results obtained by either or both methods from 120 specimens is 18, that both methods yielded positive results in 12 of these specimens and that a positive result was obtained by the sodium hydroxide method from 4 specimens in which the corresponding result by the sulphuric acid was negative whereas the reverse was the case with two specimens. This difference in efficiency of the two methods is less than that shown on the basis of patients who yielded a positive culture (table 61). Indeed it is difficult to believe that this analysis has been made from exactly the same specimens as those which, examined on the basis of patients who yielded positive results, showed that

the sodium hydroxide method produced a positive result in 3 patients in whom the result by the sulphuric acid method was negative whereas the sulphuric acid method failed to yield any positive results which were not also obtained by the sodium hydroxide method.

Contamination rates. With the sodium hydroxide method, the contamination rate was 19.2 per cent. when reckoned as the ratio of the number of slopes contaminated to the number of slopes inoculated; the contamination rate was 10.8 per cent. when reckoned as the ratio of the numbers of specimens in which no result was obtained because of contamination of both slopes inoculated from a specimen to the number of specimens examined (table 63). The corresponding figures for the sulphuric acid method were 7.1 per cent. and 1.7 per cent. respectively. The conditions of the investigation therefore favoured the sulphuric acid method, but it must be emphasised that in no instance was a positive result obtained by one method where both of the corresponding cultures by the other method were destroyed by contamination.

In this experiment the modified sulphuric acid method failed to yield better results than the sodium hydroxide method and therefore the sodium hydroxide method was adopted as the standard method for the purposes of establishing the efficiency of other methods investigated in this thesis.

Experiment 6.

The action of selective bacteriostatic and bactericidal agents on tubercle bacilli.

This study was made for the following reasons:- 1) because these agents might be used alone or in combination with standard methods in order to decontaminate sputum before culture for tubercle bacilli - an approach suggested by the work of Saxholm (1954 - see p. 31 of this thesis); 2) because it might be possible to use these agents in a selective medium which could be used for the direct isolation of tubercle bacilli from contaminated material without preliminary decontaminating procedures; and 3) because, even if it proved impossible to evolve a medium which would suppress contaminants completely and permit good growth of tubercle bacilli, it might be possible to devise a fluid enrichment medium for the isolation of tubercle bacilli from contaminated material. The efficiency of standard methods might be improved by the use of a fluid enrichment medium in the following way: if the growth of contaminants were retarded, very small numbers of tubercle bacilli might multiply and, after a suitable incubation period, the numbers of tubercle bacilli might be sufficient to permit the application of a

decontaminating agent without the risk of killing all the tubercle bacilli.

The bacteriostatic agents investigated and the concentration of these agents which permitted free growth of the H37Rv strain are shown in table 64. The concentrations of ethyl violet, proflavine, malachite green and silver nitrate which allow free growth of the H37Rv strain of tubercle bacilli might be expected to have a worth-while bacteriostatic effect on organisms other than tubercle bacilli. It must be empnasised that these results have been used only as a rough guide to the properties of the agents studied and that a detailed study of these and other bacteriostatic agents used in the experiments described in this thesis was not undertaken. Such a study would in itself provide meterial for a separate thesis.

Experiment 7.

The isolation of tubercle bacilli from sputum by means of combinations of pancreatin with bacteriostatic agents.

Saxnolm (see pages 31 to 32 and tables 30 to 34 of this thesis) claimed good results in the isolation of tubercle bacilli from sputum by means of a combination of pancreatin and Desogen. A small preliminary trial was therefore conducted (see p. 81 of this thesis) in order to assess methods of isolating tubercle bacilli in which combinations of pancreatin with Desogen and other bacteriostatic agents were used for homogenising and decontaminating sputum. The following combinations were tested:-

1) Pancreatin - malachite green.
2) Pancreatin - ethyl violet.
3) Pancreatin - proflavine.
4) Pancreatin - hibitane.
5) Pancreatin - desogen.
6) Pancreatin - silver nitrate.
7) Pancreatin - mercuric chloride.

The results are summarised in table 65; the following points deserve emphasis:-

1) Combinations of pancreatin with malachite green, ethyl

violet, Hibitane, silver nitrate or mercuric chloride failed to give satisfactory control of contamination.

2) The positive results obtained with combinations of pancreatin with Desogen or proflavine were obtained after an incubation period of 25 days and the numbers of colonies obtained from the specimens were small. The sodium hydroxide method usually yields a confluent growth of tubercle bacilli from the type of specimen used for this experiment (see p. 81 of this thesis). There was therefore no indication that Saxholm's pancreatin-desogen method, or any of the other methods tested, deserved a more thorough trial in comparison with the standard method.

Experiment 8.

The isolation of tubercle bacilli from sputum with combinations of different concentrations of trisodium phosphate with bacteriostatic agents.

For the isolation of tubercle bacilli from sputum, trisodium phosphate is usually employed in a concentration of 10 per cent. of the anhydrous salt. In the trial conducted by the Public Health Laboratory Service (1952) the trisodium phosphate method had the lowest efficiency and the highest contamination rate of the four methods investigated (see table 2 and page 13 of this thesis). Better results were obtained by Gifford et al. (see p. 18 of this thesis) who used a shorter time of exposure to trisodium phosphate and by Starkey and Aubert (see p. 17 of this thesis) and by Peizer et al. (see p. 18 of this thesis) who used a lower final concentration of trisodium phosphate; in an attempt to ensure control of contamination it was decided to test the decontaminating effects of different concentrations of trisodium phosphate combined with Hibitane or proflavine.

This experiment was designed as a preliminary trial (see p. 81 of this thesis). Four specimens of sputum were examined and each specimen of sputum was divided into equal portions; a separate portion of each sputum was prepared for culture by treatment with a different concentration of trisodium phosphate or combination of trisodium phosphate

with 0.01 per cent. Hibitane or 0.01 per cent. proflavine. The following concentrations of trisodium phosphate (anhydrous salt) were used: 10 per cent., 5 per cent., 1 per cent. either alone or in combinations with Hibitane or proflavine.

The results are summarised in table 66. All the combinations of proflavine with trisodium phosphate failed to yield positive results from any of the specimens of sputum. On the other hand, positive results were obtained with combinations of Hibitane with trisodium phosphate but only one combination - Hibitane with 1 per cent. trisodium phosphate - yielded as many positive results as trisodium phosphate without Hibitane. In this instance, growth from the specimens treated with trisodium phosphate plus Hibitane was neither earlier nor more abundant than that obtained from specimens treated with trisodium phosphate above.

It did not seem profitable to pursue this investigation further. Nevertheless, Patterson et al. (see p. 33 of this thesis) recommend the use of trisodium phosphate combined with benzalkonium chloride for the isolation of tubercle bacilli from sputum; experiment 8 was done before the work of Patterson et al. was published.

Experiment 9.

The use of Teepol in the isolation of tubercle bacilli from sputum.

The favourable results obtained by Browning (see p. 30 of this thesis) and by Tison (see p. 31 of this thesis) suggested that this agent should be investigated.

Browning's method. A small preliminary trial (see p. 81 of this thesis) was made with 5 specimens of sputum known to contain acid-fast bacilli. Four positive results were obtained within 21 days but in the slopes inoculated with a pasteur pipette, growth was poor near the water of condensation which contained a high concentration of Teepol. This confirmed 1) that Teepol exerts an inhibitory effect on tubercle bacilli - a fact established by Tison (1954) and 2) that the removal of Teepol from the inoculum by washing and centrifugation would be desirable for the growth of small numbers of tubercle bacilli. There was complete control of contamination in the specimens prepared for culture by Browning's method. For this reason it was thought that a further trial of a method involving the use of Teepol would be worth while. The method chosen for further trial is a simplification of that described by Tison and is described on p. 101 of this thesis; a mixture containing 25 per cent. Teepol and 1 per cent. sodium hydroxide is used to homogenise

and decontaminate the sputum.

The sodium hydroxide - teepol method. The efficiency of this method was assessed by comparing it with the sodium hydroxide method; the critical procedure described on p. 82 of this thesis was adopted. Three replicate specimens were examined from 15 patients - a total of 45 specimens. Each specimen was examined by the sodium hydroxide method and by the sodium hydroxide-teepol method. The number of patients (15) investigated in this critical comparison was less than the number investigated in other critical comparisons made in this thesis. The reason for this is that the experiment was terminated as soon as it became apparent that the sodium hydroxide-teepol method was certainly no more efficient than the sodium hydroxide method.

The results are presented in 2 ways as in experiment 5 (p. 139of this thesis).

Patients who yielded a positive culture. Seven of the 15 patients yielded a positive culture by either or both methods in 1 or more of 3 replicate specimens. Table 67 shows the results of culture of all 3 replicate specimens contributed by these 7 patients. The following points emerge:-

1) The sodium hydroxide method yielded a positive culture from 6 of the 7 patients (2, 3, 4, 5, 6 and 7) in at least 1 of the replicate specimens whereas the sodium hydroxide-teepol method yielded a positive culture from 4 of the 7 patients

(1, 2, 4, and 5).

2) Patient no. 1 yielded the only positive result obtained by the sodium hydroxide-teepol method but not by the sodium hydroxide method; in the only specimen from this patient from which a culture was obtained the cultures set up by the sodium hydroxide method were both destroyed by contamination.

Specimens which yielded a positive culture. Table 68 shows that of the 45 specimens 11 yielded a positive result by either or both methods: 7 specimens were positive by both methods, 3 were positive only by the sodium hydroxide method and 1 only by the sodium hydroxide-teepol method.

Contamination rates. The contamination rates of the 2 methods are compared in table 69. The contamination rate for the sodium hydroxide-teepol method (10 per cent. of all slopes inoculated) is lower than that of the sodium hydroxide method (26.7 per cent. of all slopes inoculated). Even more striking is the fact that contamination of both of the slopes inoculated from a specimen was not encountered with the sodium hydroxide-teepol method whereas 15.6 per cent. of specimens treated with the standard sodium hydroxide method failed to yield any information about the presence or absence of tubercle bacilli because of contamination of both of the slopes inoculated from each of these specimens.

Nevertheless, despite the fact that a very high

incidence of contamination with the standard sodium hydroxide method biassed the results in favour of the sodium hydroxide-teepol method, the standard sodium hydroxide method was as efficient, if not more efficient, than the sodium hydroxide-teepol method. It was therefore decided that there would be little purpose in a more extensive investigation of this simplified sodium hydroxide-teepol method which has no advantage over the sodium hydroxide method in simplicity or safety. It must be emphasised, however, that Tison's original method (see p. 31 of this thesis) was not investigated in experiment 9 and that the results of experiment 9 do not invalidate his claim that tubercle bacilli may be isolated from sputum more efficiently by his procedure involving the use of sodium hydroxide and Teepol than by the sodium hydroxide method. Tison's original method, however, is more complicated and more dangerous to the operator than the sodium hydroxide method.

Experiment 10.

The importance of centrifugation in the sodium hydroxide method.

Centrifugation is a dangerous and time-consuming procedure and is regarded as a necessary treatment for the washing and concentration of the inoculum in the standard methods of isolating tubercle bacilli from sputum. The results of Klein et al. (see p. 49 of this thesis) Hata et al. (see p. 49 and table 48 of this thesis) and Saxholm (see p. 31 and tables 30 to 34 of this thesis) suggest that centrifugation of homogenates contributes little to the efficiency of methods of isolating tubercle bacilli from sputum. Because these authors failed to use sputum known to contain very few tubercle bacilli it was decided to compare the results of culturing homogenates of sputum without concentration by centrifugation with the results of culturing the deposits produced by centrifuging the homogenates. The sodium hydroxide method of homogenisation was chosen for this experiment; 74 specimens negative for acid-fast bacilli by microscopy were investigated.

The results are summarised in table 70. A positive result was obtained from 19 of the specimens and of these

positive results, 8 were obtained only after centrifugation. Inoculation from the neutralised uncentrifuged homogenate failed to yield any positive results which were not also obtained from the centrifuged homogenate. The results suggest that in preparing sputum for culture by the sodium hydroxide method centrifugation may not be omitted without a serious loss of efficiency. The contamination rate for homogenates inoculated without centrifugation, 14.2 per cent. of all slopes inoculated, was less than that for the homogenates which were centrifuged before inoculation, 18.9 per cent. of all slopes inoculated; the numbers of specimens for which no results were obtained because both slopes were contaminated was the same for each procedure (table 71).

Experiment 11.

Fluid media for the isolation of tubercle bacilli from sputum.

The results of experiment 10 show that culture on solid medium of small portions of the neutralised homogenates of specimens of sputum yield results which are markedly inferior to those yielded by culture of the deposit obtained by centrifuging the homogenate. Therefore, it was thought that the simplest method of omitting centrifugation without loss of efficiency would be to culture the whole specimen. Two methods based on this principle were evolved. In the first, untreated sputum was cultured in the fluid medium EV (see p. 74 of this thesis) and, in the second, sputum homogenised with 1 per cent. sodium hydroxide was cultured in the fluid medium PF (see p. 74 of this thesis) without preliminary concentration by centrifugation.

Culture of untreated sputum in EV medium. Specimens of sputum were divided into 2 equal portions; one portion was cultured by the sodium hydroxide method and the other was transferred without preliminary decontamination into EV medium. Thirty-two specimens were examined; of these, 7 were found to contain a few acid-fast bacilli only after concentration with sodium hydroxide and the remaining 25

specimens were negative for acid-fast bacilli even after concentration.

The results are summarised in table 72. With the 7 specimens positive for acid-fast bacilli only after concentration, the EV medium yielded 3 positive results compared with 6 positive results obtained by the sodium hydroxide method. With the 25 specimens completely negative for acid-fast bacilli by microscopy, the EV medium failed to yield any positive results whereas the sodium hydroxide method yielded 5. It is clear that culture of untreated sputum in EV medium is much less efficient than culture by the sodium hydroxide method and it must be emphasised that all the EV cultures, including those in which micro-colonies of tubercle bacilli were observed, were heavily overgrown with contaminants. It is probable that failure to control contamination is the main reason why this method compares so unfavourably with the sodium hydroxide method.

Culture of homogenised sputum in P.F. medium.

Failure of the EV medium to control contamination in the culture of untreated sputum suggested that preliminary homogenisation and decontamination with 1 per cent. sodium hydroxide followed by culture in PF medium of all of the homogenate might yield better results. Twenty-seven specimens of sputum were divided into 2 equal portions; one

portion was homogenised with 1 per cent. sodium hydroxide and the whole homogenate was cultured in the PF medium; the other portion was cultured by the standard sodium hydroxide method. The buffering action of the PF medium was such that, after the addition of the alkaline homogenate, the pH did not rise above 7.5. In 3 of the specimens, acid-fast bacilli were detected by direct microscopy; in a further 5, a few acid-fast bacilli were detected after concentration by the sodium hydroxide method; and in the remaining 19 specimens acid-fast bacilli were not detected even after concentration.

The results are summarised in table 73. The PF medium yielded only 1 positive result from the 3 specimens positive by direct microscopy; 3 positive results were obtained from these specimens by the sodium hydroxide method. From the 5 specimens positive only after concentration, 2 positive results were obtained in PF medium and 5 by the sodium hydroxide method. In the 19 specimens negative even after concentration the PF medium failed to yield any positive results whereas the sodium hydroxide method yielded 4 positive results. All of the cultures in the PF medium including the positive ones were overgrown by contaminants. Thus, despite preliminary decontamination of the sputum and the incorporation of bacteriostatic agents in the medium, fluid cultures from sputum in PF medium are readily overgrown by

contaminants; this is probably the main reason why the medium compared unfavourably with the sodium hydroxide method.

Experiment 10 constitutes only a preliminary examination of the possible uses of fluid media for the isolation of tubercle bacilli from sputum. In my opinion, the unfavourable results do not rule out the possibility of progress towards simplicity and efficiency in the isolation of tubercle bacilli from sputum by means of fluid media. Some of the preliminary work for this experiment is reported in experiment 6 and it is pointed out on p.144 of this thesis that much more elaborate tests than those I have employed would be required in order to evolve a combination of bacteriostatic agents which would permit free growth of tubercle bacilli and inhibit the growth of contaminants. Investigation of this means of avoiding centrifugation was abandoned at this point only because it offered little immediate prospect of success.

Experiment 12.

The evaluation of Nassau's swab method of isolating tubercle bacilli.

Nassau (1954) described a method of isolating tubercle bacilli from sputum by means of cotton wool swabs. Details of the method are given on p. 107 of this thesis. The method was examined critically because it is simple and avoids the risks attached to homogenisation and centrifugation (see p. 57 of this thesis). Four groups of specimens were used to evaluate the swab method:-

1) Specimens positive for acid-fast bacilli by direct microscopy. The swab method was applied to these specimens without comparison with the sodium hydroxide method; specimens of this kind should yield a very high proportion of positive results by any reasonably efficient method.

2) Specimens negative for acid-fast bacilli by direct microscopy but positive after concentration with sodium hydroxide. The results obtained by the swab method from these specimens were compared with those obtained by the sodium hydroxide method.

3) Single specimens negative by microscopy for acid-fast bacilli even after concentration. Each specimen in this group was obtained from a different patient. The results obtained

from these specimens by the swab method were compared with those obtained by the sodium hydroxide method.

4) Replicate specimens negative by microscopy for acid-fast bacilli even after concentration. Patients who contributed specimens to this group each contributed 2 or more replicate specimens. The results obtained from these specimens by the swab method were compared with those obtained by the sodium hydroxide method. Two comparisons were made: (1) of the numbers of patients who yielded a positive result from one or more replicate specimens and (2) of the numbers of specimens which yielded a positive result.

The following results were obtained.

Specimens positive for acid-fast bacilli by microscopy. As shown in table 74 tubercle bacilli were isolated by the swab method from 92 of 96 specimens. Although no comparison was made with these sputa between the swab and sodium hydroxide methods, it is clear that the results with the swab method leave little room for improvement. The contamination rate was satisfactorily low.

Specimens negative for acid-fast bacilli by direct microscopy but positive after concentration with sodium hydroxide. In this small group of 30 specimens there was no striking difference in efficiency between the swab method and the sodium hydroxide

method. Table 75 shows that the swab method yielded 1 culture of tubercle bacilli when the corresponding culture by the sodium hydroxide method was negative and the sodium hydroxide method yielded 5 cultures of tubercle bacilli when the corresponding cultures by the swab method were negative. Single specimens negative by microscopy for acid-fast bacilli even after concentration with sodium hydroxide. This group of specimens consisted of 130 specimens - one from each of 130 patients. The efficiency of the swab method is compared with that of the sodium hydroxide method in table 76. Both methods yielded cultures from 19 of the specimens; the swab method yielded cultures of tubercle bacilli from 5 specimens which gave negative results by the sodium hydroxide method; and the sodium hydroxide method yielded cultures of tubercle bacilli from 12 specimens which gave negative results by the swab method. When the contamination rate is based on the number of specimens for which a result was not obtained because of contamination of both slopes inoculated from these specimens, the rate for the sodium hydroxide method, 10 per cent., is 4 times that for the swab method, 2.5 per cent. (table 77). Because of the relatively high contamination rate with the sodium hydroxide method the comparison of the two methods was biased in favour of the swab method. Omitting the specimens in which the result of culture by either method is influenced by contamination, the difference between the two methods in favour of the sodium hydroxide method is even more striking (table 76).

Replicate specimens negative by microscopy for acid-fast bacilli even after concentration with sodium hydroxide. The second group of specimens in which acid-fast bacilli were not seen even after concentration was obtained from 41 patients; 2 or more replicate specimens were obtained from each patient and the total number of specimens in the group is 104. The interval between the examination of replicate specimens from a particular patient was from 3 to 10 days.

Patients who yielded a positive culture. Table 78 gives details of the results obtained from 19 patients who yielded a positive culture by either or both methods in 1 or more of the replicate specimens which they contributed. The following points emerge:-

1) The sodium hydroxide method failed to yield a positive result in only 1 instance (patient 8); on the other hand the swab method failed to yield a positive result in 10 instances (patients 1, 3, 4, 5, 7, 9, 10, 12, 13 and 19).

2) When the specimens are divided into 3 separate series of of single specimens according to the chronological order in which each specimen from each patient was examined, the success rate for the swab method in each series is only half that of the sodium hydroxide method.

3) In the only patient in whom the swab method alone was successful, the specimen which yielded the positive result by the swab method may have failed to yield a positive result by the sodium hydroxide method because both slopes inoculated

by this method were contaminated.

Specimens which yielded a positive culture. Table 79 shows the numbers of specimens which yielded positive results in 104 specimens taken from 41 patients. As would be expected from comment (2) above on p.162 of this thesis, the success rate for the swab method is only half that of the sodium hydroxide method.

These results show that although the swab method is highly efficient with specimens known to contain acid-fast bacilli (tables 74 and 75) its efficiency compares very unfavourably with that of the sodium hydroxide method with specimens in which microscopy failed to demonstrate acid-fast bacilli.

The simplicity and safety of the swab method make it very attractive. It is highly efficient with specimens known from microscopy to contain acid-fast bacilli and this fact gives the method a useful place in routine laboratory practice. The danger of creating highly infective aerosols by centrifugation and shaking can be greatly reduced by culturing microscopically positive specimens by the swab method and by limiting to microscopically negative specimens the application of the more efficient but more dangerous and time-consuming sodium hydroxide method.

Experiment 13

Comparison of sputum culture and laryngeal swab culture for the isolation of tubercle bacilli from patients suffering from pulmonary tuberculosis.

The results of experiment 12 show that it is possible to reduce the danger of laboratory infection by (a) adopting Nassau's swab method for specimens known from microscopy to contain acid-fast bacilli and (b) reserving the sodium hydroxide method for specimens in which direct microscopy fails to detect acid-fast bacilli. This arrangement is not ideal because it involves the operation of 2 methods in 1 laboratory. Laryngeal swabs may be prepared for culture in exactly the same way as sputum swabs (see p.107 of this thesis). It was therefore decided to compare the efficiency of laryngeal swab culture with that of sputum culture in patients whose sputum was negative by microscopy for tubercle bacilli because, if laryngeal swab culture compared favourably with culture from sputum by the sodium hydroxide method, a simple, safe, efficient and uniform method could be adopted in routine laboratory practice.

Forty-six patients, all of whom produced sputum which was negative by microscopy for acid-fast bacilli were investigated.

Each patient contributed 3 replicate specimens for culture by the sodium hydroxide method and when each specimen was collected for culture, laryngeal swabs were taken. In this way the result of culture of each specimen of sputum, taken at a given time, was compared with the result of culturing 2 laryngeal swabs taken at approximately the same time. The time over which replicate specimens were taken from a given patient was never more than 10 days.

Patients who yielded a positive culture. Thirteen of the 46 patients yielded a positive culture by either or both methods from 1 or more of the replicate specimens. Table 80 shows the results obtained in 3 replicate specimens contributed by each of the 13 patients who yielded a positive culture. The following points emerge:-

1) The sodium hydroxide method yielded at least 1 positive culture in all of the 13 patients whereas the laryngeal swab method failed to yield a positive culture in 4 patients (2, 4, 8 and 13).

2) When the specimens are divided into 3 separate series according to the chronological order in which each specimen from each patient was examined, the difference between the 2 methods is slight in the first and third series but very marked in the second series.

3) In 2 instances in which a positive result was obtained from a single specimen only by the laryngeal swab method, the

corresponding cultures by the sodium hydroxide method were spoiled by contamination (patient 6, third replicate specimen; patient 12, first replicate specimen). In 1 instance the reverse was the case (patient 12, second replicate specimen).

Specimens which yielded a positive culture. Table 81 shows that of 138 specimens examined 33 yielded a positive result by either or both methods. A positive result was yielded by both methods in 12 specimens; a further 16 positive results were obtained only by the sodium hydroxide method and a further 5 only by the laryngeal swab method.

The results of this investigation show that, however these results are analysed, the sodium hydroxide method is superior to the laryngeal swab method and that although replicate examination of specimens increased the yield of positive results from the 46 patients by the laryngeal swab method, a greater increase was obtained when the sodium hydroxide method was employed.

Contamination rates. The ratio of the number of contaminated slopes to the number of slopes inoculated was 62/276 (22.5 per cent.) for the sodium hydroxide method and 24/276 (8.6 per cent.) for the laryngeal swab method (table 82). Because only 2 slopes were inoculated by each method from a given examination, contamination of both slopes in one examination ruled out the possibility of obtaining a positive result. This was the case in 12.3 per cent. of examinations by the

sodium hydroxide method and 1.4 per cent. of examinations by the laryngeal swab method. The superiority of the sodium hydroxide method in this investigation is therefore demonstrated under conditions which favoured the laryngeal swab method.

These results suggest that, although in a single series of specimens the laryngeal swab method may yield a positive result in a few specimens in which the corresponding result by the sodium hydroxide method is negative (table 80, first and third groups of replicate specimens), the laryngeal swab method is not efficient enough to replace the sodium hydroxide method.

Another feature of laryngeal swab specimens must also be mentioned; they can be prepared for culture by a method which is relatively safe for the laboratory worker but they might well be a source of danger to the person who takes the swabs from the patient. Therefore the general application of the laryngeal swab method would simply transfer a risk from the laboratory worker to the clinical worker.

Experiment 14

Concentration of tubercle bacilli from homogenates of sputum by sedimentation with barium sulphate.

So far, in these experiments, it has not been possible to avoid centrifugation and retain efficiency in the isolation of tubercle bacilli from sputum; the following approaches to the problem have failed to produce a satisfactory alternative to the use of centrifugation: 1) the cultivation of a small portion of neutralised homogenate without preliminary centrifugation (experiment 10, p. 158 of this thesis); 2) the use of a fluid medium for the cultivation of either a complete specimen of sputum or the total volume of the homogenised specimen (experiment 11, p. 155 of this thesis); 3) the preparation of sputum for culture by means of cotton wool swabs (experiment 12, p.159 of this thesis); and 4) the culture of laryngeal swab specimens (experiment 13, p. 164 of this thesis). The present experiment was designed to demonstrate whether tubercle bacilli could be concentrated efficiently from a sputum homogenate by adding a suspension of barium sulphate to the homogenate; the barium sulphate suspension adheres to the more solid particles of the homogenate and, without centrifugation, a deposit is rapidly formed which may be used for inoculating media.

A simple procedure is described on p. 114 of this thesis for the concentration of tubercle bacilli from sputum homogenates by means of barium sulphate; it involves the use of special sedimentation tubes (illustrated in figs. 4, 5 and 6 and described on p. 113 of this thesis).

The efficiency of barium sulphate sedimentation was compared with that of centrifugation in two separate series of specimens. The first series was small and used as a preliminary test in order to demonstrate whether the method could concentrate small numbers of tubercle bacilli from sputum; the second series was larger than the first and was used to ensure a more critical comparison of the efficiency of barium sulphate sedimentation and centrifugation.

Preliminary examination of the efficiency of barium sulphate sedimentation. Fifteen patients were investigated; each specimen was homogenised with sodium hydroxide and the homogenate was divided into 2 equal portions. The first portion was washed and concentrated by centrifugation in the usual way (see p. 74 of this thesis) and the second was washed and concentrated by means of barium sulphate (see p. 114 of this thesis).

The results show 1) that concentration with barium sulphate compares favourably with centrifugation (tables 83 and 84) and 2) that the contamination rate with barium sulphate concentration is no greater than with centrifugation (table 85). The

results of culture by both methods in the second replicate specimen from patient 4 are illustrated in figures 7A and 7B. It is clear that barium sulphate sedimentation concentrated the tubercle bacilli from this specimen as efficiently as centrifugation. These preliminary results indicated that further trial of the method was worth while.

Critical examination of barium sulphate sedimentation. In this part of the experiment it was decided to compare the efficiency of centrifugation of homogenates prepared by the sodium hydroxide method with the efficiency of barium sulphate sedimentation of homogenates prepared from the same specimens of sputum by the method of Patterson et al. (1956, see p. 33 of this thesis). The reasons for adopting the method of Patterson et al. for the preparation of homogenates for concentration by barium sulphate sedimentation are as follows. First, Patterson et al. claim that their method of decontaminating sputum is more efficient than the sodium hydroxide method (table 36 and p. 33 of this thesis); second, they claim that their method has a much lower contamination rate than the sodium hydroxide method; and third, their method can readily be adapted for concentration by barium sulphate (see p. 115 of this thesis).

Fifty-two patients, all of whom produced sputum which was negative by direct microscopy for acid-fast

bacilli were investigated. Each patient contributed 3 replicate specimens and an equal portion of each specimen was cultured by each method. In other words, 156 specimens were cultured by the sodium hydroxide method (homogenate concentrated by centrifugation) and by a modification of Patterson's method (homogenate concentrated by barium sulphate sedimentation). The results are summarised in tables 86, 87 and 88.

Patients who yielded a positive culture. Eighteen of the 52 patients yielded a positive culture by either or both methods in 1 or more replicate specimens. The results of culture in all 3 replicate specimens from each of these 18 patients are presented in detail in table 86. The following points emerge:-

1) Three of the patients (3, 6, 15) failed to yield a positive culture by the sodium hydroxide method with centrifugation whereas 6 patients (2, 7, 9, 10, 16, 18) failed to yield a positive culture by Patterson's method with barium sulphate sedimentation.

2) When the specimens are divided into 3 separate series of specimens according to the chronological order of examination of each specimen from each patient, the difference between the 2 methods is always in favour of the sodium hydroxide method with centrifugation.

Specimens which yielded a positive culture. Table 87 shows that 35 positive results were obtained from 156 specimens by either or both methods; that both methods yielded positive results in 18 specimens. A positive result was obtained by the sodium hydroxide method with concentration by centrifugation in 14 specimens in which the corresponding result was negative by Patterson's method with concentration by barium sulphate sedimentation; the reverse was the case in only 3 specimens.

Contamination rates. Table 88 shows that for the sodium hydroxide method with centrifugation the proportion of the total number of slopes spoiled by contamination (16.7 per cent.) is approximately the same as that for Patterson's method with barium sulphate sedimentation (14.1 per cent.). On the other hand, with Patterson's method with concentration by barium sulphate sedimentation, the proportion of specimens in which culture yielded no result because of contamination of both slopes inoculated was 5.8 per cent. whereas the corresponding figure for the sodium hydroxide method with centrifugation was 9 per cent. The control of contamination by Patterson's method in this experiment was much less efficient than that originally reported by Patterson et al. (see tables 36 and 37).

Barium sulphate sedimentation is less efficient than

centrifugation for the concentration of tubercle bacilli from sputum homogenates. The use of barium sulphate sedimentation is safer and simpler than centrifugation. However, the efficiency of Patterson's method modified by substituting barium sulphate sedimentation for centrifugation (tables 86 and 87) is of the same order as that of an even simpler method - the culture of laryngeal swabs (tables 80 and 81). Barium sulphate sedimentation used as described in this experiment (see p. 114 of this thesis) has therefore no place in the routine isolation of tubercle bacilli from sputum.

GENERAL DISCUSSION

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The experiments which I have described in the previous section are best regarded as a preliminary study of methods of isolating tubercle bacilli from sputum; the reasons for this opinion are as follows. The information which I have obtained does not establish any one method as wholly satisfactory; it does not even permit me to recommend any one method as the best available for all specimens of sputum. The most efficient method - the sodium hydroxide method - is not safe enough to be applied routinely to specimens containing large numbers of tubercle bacilli; the safest method - Nassau's (1954) swab method - is not efficient enough to be applied routinely to specimens likely to contain very few tubercle bacilli. The following topics will therefore form the basis of the discussion of my experimental work:

1) The validity of the results.

2) The information yielded by the experiments.

3) Unsatisfactory experiments.

4) Future work required to establish a safe, simple and efficient method of isolating tubercle bacilli from sputum.

THE VALIDITY OF THE RESULTS

Two main procedures have been used to examine methods of isolating tubercle bacilli from sputum: 1) quantitative studies based on viable counting and 2) studies with series of sputum in which the efficiency of a given method was compared with that of a standard method - the sodium hydroxide method. Before discussing the results yielded by the two procedures it is worthwhile to assess the value of the information yielded by each procedure.

Quantitative studies.

Two questions must be answered in order to assess the value of viable counting as a means of comparing the efficiency of methods of isolating tubercle bacilli from sputum. First, have results obtained by viable counting from specimens containing large numbers of tubercle bacilli any relevance for the most important specimens dealt with under routine conditions - specimens likely to contain very few tubercle bacilli? Second, in a comparison by means of viable counting, what difference between 2 methods is to be regarded as significant?

The first question cannot be answered satisfactorily

because, in specimens containing few tubercle bacilli, it is doubtful whether the vitality of the organisms is as great as that of organisms in specimens containing large numbers of tubercle bacilli. However, even if this theoretical consideration is ignored, the results of experiment 1 (table 55) show that it would be unprofitable to attempt to draw general conclusions which would apply under routine conditions because the efficiency of any one method differs from specimen to specimen.

It is worth noting at this point that the unpredictable nature of the lethal effect of a decontaminating agent was pointed out by Griffith as early as 1916 (see p. 28 of this thesis). Griffith, however, correlated differences in lethal effect of antiformin with the "thickness" or "thinness" of the specimen to which his method was applied; the specimens examined in experiment 1 were all "thick" ones - that is, purulent specimens of sputum. Although Spendlove et al. (see p. 28 of this thesis) emphasise that factors which viable counting does not assess may well account for differences between the results of studies with viable counting methods and those of studies with series of specimens of sputum, they concluded, on the basis of experiments (table 22) with pooled sputum from tuberculosis patients, that sputum did not minimise

the lethal effect of decontaminating agents. Gray et al. (see p. 28 of this thesis) also maintain that sputum did not influence the lethal effect of decontaminating agents; in support of this view they give the results of an experiment (table 24) in which known numbers of a laboratory strain of tubercle bacilli suspended in purulent non-tuberculous sputum were treated with decontaminating agents. In experiment 1 (table 55) I investigated 7 specimens of tuberculous sputum each obtained from a different patient. Therefore, unlike Spendlove et al. and Gray et al. I provide worth-while evidence in support of my conclusion concerning the influence of sputum on the lethal effect of decontaminating agents; this evidence makes it clear that all the tubercle bacilli in one specimen of sputum may survive the action of sodium hydroxide (table 55, specimen 1) whereas less than one-seventh of the tubercle bacilli in another specimen may survive (table 55, specimen 5). Therefore, it must be concluded either that tubercle bacilli differ in susceptibility to sodium hydroxide from one specimen to another or that sputum may inhibit or augment the lethal effect of sodium hydroxide.

In the light of the above considerations, the answer to the second question is of relatively little importance. However, even if the lethal effect of the

decontaminating agents studied in experiment 1 were constant from specimen to specimen, I think that my viable counting procedure or any other known to me is not sufficiently sensitive to demonstrate a clear difference in efficiency between the sulphuric acid method and the sodium hydroxide method - that is, a difference as convincing as that shown by the Public Health Laboratory Service (table 2) or even that shown in experiment 5 (table 61).

Two further points must be raised concerning the validity of my results with quantitative studies. First, the results of experiment 1 (table 56) and of experiment 4 (table 60) show that centrifugation is an efficient means of concentrating tubercle bacilli from homogenates of sputum; this conclusion has been verified and, in my opinion, established more clearly by results obtained in a study with a series of specimens of sputum (table 70). Second, the results of experiment 3 (tables 58 and 59) suggest that neutralisation may have a lethal effect on the tubercle bacilli present in homogenates of sputum prepared by the sodium hydroxide method. In retrospect, I believe that the importance of the lethal effect of neutralisation could have been demonstrated much more clearly in a study with a series of specimens of sputum.

It must be concluded, therefore, that my experiments with viable counting show that the advantages claimed on p.119 of this thesis for this method of studying decontaminating agents are largely theoretical and that viable counting has no place in the critical assessment of methods of isolating tubercle bacilli from sputum. Results obtained with aqueous suspensions of tubercle bacilli (Spendlove et al., Gray et al.) or with pooled sputum (Spendlove et al.) or with laboratory strains of tubercle bacilli suspended in purulent non-tuberculous sputum (Gray et al.) are not only of little value but may lead to erroneous conclusions.

Studies with specimens of sputum.

When methods of isolating tubercle bacilli from sputum are compared in a series of specimens, the validity of the results depends 1) on the selection of the specimens and 2) on the numbers of specimens examined. The study conducted by the Public Health Laboratory Service (p. 13 to 15 of this thesis; table 2) is a model of the correct procedure - the specimens were properly selected and large numbers of specimens were examined.

Selection of specimens. The specimens used for the comparisons made in experiments 5, 9, 10, 11, 12, 13 and 14 were selected very carefully - they were obtained from known cases of

pulmonary tuberculosis and were negative for acid-fast bacilli by direct microscopy. Moreover, in some of the experiments (5, 9, 12, 13 and 14), replicate specimens from a relatively small number of patients were used instead of a larger series of specimens, each obtained from a different patient. A chest-physician attaches little importance to a negative result from a single specimen of sputum; therefore, a negative report on a single specimen usually leads to the submission of 2 or more further specimens from the same patient. Accordingly, routine clinical and laboratory practice is followed very closely by examining replicate specimens from patients whose sputum is negative by microscopy for acid-fast bacilli. I believe, therefore, that I have chosen for my critical comparisons the material most likely to give a true estimate of the efficiency of a given method under the conditions in which the method would normally be applied.

Number of specimens. The numbers of specimens (120 to 150) used in my critical comparisons were small compared with those used by the Public Health Laboratory Service (for each comparison approximately 1,200 examinations were made of sputum negative by microscopy for acid-fast bacilli). As already stated on p.83 of this thesis, I could not examine or even obtain numbers of the correct type of specimen as large as those examined in the Public Health Laboratory Service trial for which the resources of 12 laboratories were available.

The value of replication. Despite the fact that I could examine only relatively small numbers of specimens, I must emphasise that I did succeed in showing important differences between methods. The smallest difference which I found in a critical comparison of 2 methods is that demonstrated in experiment 5 - a comparison of the efficiency of the sodium hydroxide method with that of a modified sulphuric acid method. Nevertheless, the results of experiment 5 show clearly the value of examining replicate specimens of sputum. Table 62 suggests that, on the basis of the numbers of positive results yielded by each method from 120 specimens of sputum, there is no significant difference between the 2 methods. On the other hand, on the basis of the numbers of positive results obtained from the 40 patients from whom the specimens were obtained table 61 shows that the sodium hydroxide method yielded a positive result in 3 patients in whom a negative result was obtained by the sulphuric acid method whereas the sulphuric acid method failed to yield a positive result in any patient in whom a negative result was obtained by the sodium hydroxide method. In any one of these 40 patients the following clinical problems might arise: is this patient's minimal lesion a tuberculous one? is this patient's tuberculous lesion active? is this patient infective? if this patient returns to full employment will his lesion break down? has this patient received the maximum benefit of

chemotherapy? is there an indication for surgery in this patient and, if so, when should surgical treatment be undertaken? The answers to these problems depend, at least to an important degree, on whether tubercle bacilli can be demonstrated in the patient's sputum and the results shown in table 61 indicate that, if the sulphuric acid method had been used alone, the wrong answer might have been given in 3 patients even after repeated examination of the sputum.

The value of proper selection of specimens. Apart from the use or replicate specimens - an approach to the assessment of the efficiency of decontaminating procedures described for the first time (O'Hea (1957)) - I have also shown that the proper selection of specimens as recommended by the Public Health Laboratory Service (see p. 13 of this thesis) may yield important information even with very few specimens. The results of experiment 10 (table 70) show that centrifugation may not be omitted from the sodium hydroxide method without serious impairment of the efficiency of the method; only 74 specimens of sputum were examined in this experiment, but they were specimens likely to contain only very small numbers of tubercle bacilli. This result contradicts those of Klein et al. (p. 49 of this thesis) and those of Hata et al. (p. 49 of this thesis; table 48). These authors suggest that centrifugation

contributes little to the efficiency of the sodium hydroxide method but their observations were not made on specimens containing only small numbers of tubercle bacilli; I must emphasise that if I had examined specimens of the wrong type - even 7,400 of them - I, too, would have been led to the same erroneous conclusion.

I have no hesitation, therefore, in claiming that, on the basis of the specimens selected and of the numbers of specimens examined. My procedure for experiments 5, 9, 10, 11, 12, 13 and 14 was the correct one for the circumstance in which these experiments were carried out. Comparison of the material used in these experiments with the material used by other workers makes it clear 1) that, if they had selected their material correctly, their results would have yielded much more valuable information and 2) that the careful selection of specimens is much more important than the accumulation of impressive numbers of specimens. For example, Baker (table 3), Starkey and Aubert (table 6), Gernez-Rieux et al. (table 15) and Van Vranken (table 18) attempt to compare methods despite the fact that each method was applied to a different series of specimens; Byham (table 5), Gifford et al. (table 7), Peizer et al. (table 8), Macfarlane et al. (tables 12 and 13), Madsen (table 16), Beattie (table 19), Tison and Loze (table 27), Tison (table 28),

Tison and Audrin (table 29), Saxholm (tables 30, 31, 32, 33 and 34), Lind (table 35) and Patterson et al. (tables 36 and 37) all fail to demonstrate clearly whether the methods they examined are suitable for specimens likely to contain only small numbers of tubercle bacilli.

INFORMATION YIELDED BY THE EXPERIMENTS

Studies with viable counts.

The fundamental fact established by these studies is this: viable counting is of little value for assessing the efficiency of methods of isolating tubercle bacilli from sputum (see above pages 176 to 180 of this thesis). Therefore, there is no point in discussing further the differences between my results and those of Spendlove *et al.* (see p. 25 of this thesis) or those of Gray *et al.* (see p. 26 of this thesis).

Studies with series of specimens of sputum.

I have been able to establish the following points:-

1) The sodium hydroxide method is more efficient than the sulphuric acid method (experiment 5; table 61). This result agrees with that obtained by the Public Health Laboratory Service (table 2).

2) Concentration by centrifugation is of vital importance to the efficiency of the sodium hydroxide method (experiment 10, table 70). This conclusion is the opposite of that reached by Klein *et al.* (see p. 49 of this thesis) and that reached by Hata *et al.* (table 48). For the reason stated above on p. 184 of this thesis, I believe that my conclusion is the correct one.

3) Nassau's swab method is highly efficient in the isolation of tubercle bacilli from sputum which contains numerous tubercle bacilli (experiment 12, table 74). I must emphasise the importance of this finding. The handling of specimens of sputum containing large numbers of tubercle bacilli by procedures involving shaking or centrifugation is an important source of laboratory infection (see pages 57 to 59 of this thesis). Nassau's swab method is safe and simple because it does not involve homogenisation or centrifugation; it is efficient for specimens containing large numbers of tubercle bacilli. Therefore, the serious risk in the isolation of tubercle bacilli can be greatly reduced because specimens containing numerous acid-fast bacilli need no longer be cultured by the dangerous standard methods.

4) Methods designed to eliminate centrifugation are not efficient enough for routine use in the isolation of tubercle bacilli from specimens containing very few tubercle bacilli (experiment 11, tables 72 and 73; experiment 12, tables 76, 78 and 79; experiment 13, tables 80 and 81; experiment 14, tables 86 and 87). Specimens likely to contain very few tubercle bacilli are the most important specimens dealt with in the routine laboratory investigation of tuberculosis (see above p.182 of this thesis) and it is

therefore necessary to treat these specimens by the most efficient method available at present - the sodium hydroxide method.

5) The contamination rates reported in my experiments for the sodium hydroxide method are high. If contamination is assessed by the percentage of specimens in which a positive or negative result of culture was not available because of contamination of all the media inoculated from a given specimen, the rates range from 10 per cent. (experiment 12, table 77) to 15.6 per cent. (experiment 9, table 69). These rates compare unfavourably with those reported for other methods examined in my experiments: for example, 1.7 per cent. for the sulphuric acid method (experiment 5, table 63), 2.3 per cent for Nassau's swab method (experiment 12, table 77), 1.4 per cent. for the laryngeal swab method (experiment 13, table 82) and nil for the sodium hydroxide-teepol method (experiment 9, table 69). Because it failed to control contamination satisfactorily, the sodium hydroxide method had fewer chances of yielding positive results in these experiments; nevertheless it was more efficient for the isolation of tubercle bacilli than the other methods mentioned above.

My contamination rates with the sodium hydroxide method (10 per cent. to 15.6 per cent.) are also very much

higher than those reported for this method by the Public Health Laboratory Service (4.9 per cent., table 2), Gifford et al. (1.03 per cent., table 7), Peizer et al. (2.4 per cent., table 8), Tison and Loze (3.2 per cent., table 27) and Saxholm (1.2 per cent., table 32; 1.1 per cent., table 33); on the other hand they are of the same order of magnitude as those reported by Mullahy (8 per cent., table 4), Madsen (16.9 per cent., table 16), Saxholm (15 per cent., table 30; 17.3 per cent., table 31), Lind (9.7 per cent., table 35) and Patterson et al. (13.7 per cent., table 36); and they are lower than that reported by Corper and Cohn (52 per cent., p. 22 of this thesis).

These differences between my contamination rate with the sodium hydroxide method and that reported by the Public Health Laboratory Service might be explained as follows. I inoculated 2 slopes of Löwenstein-Jensen medium from each specimen investigated whereas in the trial conducted by the Public Health Laboratory Service, 6 slopes were inoculated from each specimen; it is probable that if the inoculum is spread over a relatively large number of slopes of Löwenstein-Jensen medium, the small amount of malachite green in each slope has fewer contaminants to deal with and the general control of contamination by the medium is therefore more efficient when 6 slopes are inoculated from each specimen

instead of 2. I am unable to provide a satisfactory explanation for differences in the contamination rates for the sodium hydroxide method in my experiments and those cited by other workers (see above p. 189 of this thesis).

It might be thought that I should have attempted to achieve better control of contamination with the sodium hydroxide method by increasing the concentration of sodium hydroxide or by increasing the duration of treatment. There are 3 reasons why this was not done. First, an increased concentration of sodium hydroxide or a longer period of treatment might well have reduced not only the contamination rate but the efficiency with which tubercle bacilli were isolated. Second, the sodium hydroxide method which I have used is more efficient than any other method I have examined - even those which give more satisfactory control of contamination; therefore, it seemed unnecessary for the purposes of my experiments to devote time and labour to improvement of the control of contamination by this method. Third, the high contamination rates for the sodium hydroxide method in experiments 5, 9, 12, 13 and 14 are relatively unimportant because replicate specimens were examined from each patient and therefore it was possible to obtain from

each patient a satisfactorily large number of results which were not influenced by contamination.

UNSATISFACTORY EXPERIMENTS

Studies with the tubercle bacillus are carried out under difficulties not encountered in studies with other micro-organisms. Therefore, criticism of the experiments described in this thesis should be made in the light of these difficulties.

First, tubercle bacilli grow more slowly than other micro-organisms. Even with strains which have been maintained on artificial media, macroscopic growth rarely appears on solid media in less than 7 days; with strains which are being isolated for the first time from specimens of sputum, growth is rarely detected on solid media in less than 10 days and if only a few tubercle bacilli are present, growth may not be detected until 3 to 8 weeks from the time of inoculation.

Second, tubercle bacilli are an important source of laboratory infection. Therefore the volume of work undertaken must be limited to that which can be handled with the proper care, skill and concentration.

Third, results obtained with laboratory strains of tubercle bacilli may not be applicable to tubercle bacilli present in sputum. Even with tubercle bacilli present in sputum, results obtained with one specimen may differ markedly from those obtained by the same experimental procedures with

another specimen. It is therefore necessary to use series of specimens of sputum for most experiments; large numbers of specimens must be examined in order to select a worth-while number of the correct type of specimen.

These considerations make it clear that experiments with tubercle bacilli make heavy demands on the skill and patience of the investigator because even preliminary experiments cannot be undertaken lightly; for this reason it is often impracticable to make a wide preliminary survey of possible experimental approaches to a problem - the kind of preliminary work which does a great deal to ensure that an experiment will yield worth-while results. This explains why some experiments have been undertaken in the absence of some important preliminary information and why certain experiments give results which are suggestive rather than conclusive. The results of experiments 3, 6, 7, 11 and 14 will be discussed in the light of these considerations.

Experiment 3. The results of this experiment (tables 58 and 59) suggest that neutralisation may have an important letnal effect on tubercle bacilli contained in sputum homogenised by the sodium hydroxide method. This letnal effect was observed in only 3 of the 6 homogenates studied (table 58, specimen 1; table 59, specimens 5 and 6) and therefore the

importance of the lethal effect cannot be assessed satisfactorily. As already mentioned above on p.179 , the importance of the lethal effect could probably have been assessed more accurately by using the critical procedure described on p.82 of this thesis.

Experiment 6. This study of the influence on the growth of tubercle bacilli of different concentrations of bacteriostatic agents can only be used as a very rough guide to the action of these agents on tubercle bacilli contained in sputum. The reasons for this are as follows: 1) a laboratory strain of tubercle bacilli - the H37Rv strain - was used, 2) the inoculum was relatively large and 3) the agents were not tested in the presence of sputum which may have an important effect on their bacteriostatic properties. More detailed study of these agents is desirable; because of the magnitude of this task (see below, p.203 of this thesis), I did not persevere with these studies although I understood that the information gained in this experiment would only have a very limited application in later experiments, especially in experiment 11 (see below, p.196 of this thesis).

Experiment 7. The results of this experiment led me to believe that Saxholm's pancreatin-desogen method (see p.31 of this thesis) did not deserve a critical comparison with

the sodium hydroxide method. This contradicts the opinion I expressed in the review of the literature (see p. 66 of this thesis). I examined only 6 specimens of sputum with Saxholm's method; although I obtained a positive result with each of the 6 specimens (table 65), growth was not very abundant and took more than 25 days to appear (see p. 81 of this thesis). I must emphasise that this is hardly sufficient evidence on which to condemn the method as inefficient; I merely state that, in the light of my experience of the method with these 6 specimens, it seemed more profitable at the time to devote to other more promising methods the time available for critical experiments. My belief that the efficiency of Saxholm's method is unlikely to compare favourably with that of the sodium hydroxide method is strengthened by the results of later experiments. In experiment 10 with the sodium hydroxide method it was found that, in specimens containing few tubercle bacilli, the culture of a small portion of homogenate yielded many fewer positive results than culture of the deposit obtained by centrifuging the whole homogenate (table 70). In Saxholm's method the homogenate is not concentrated by centrifugation and only a small portion of the homogenate is cultured (4 drops from a pasteur pipette on each of 4 slopes of Lowenstein-Jensen medium). In addition, experiment 12 shows that, with specimens containing few tubercle

bacilli, culture of a small portion of sputum collected on a cotton-wool swab yields significantly fewer positive results than culture of a concentrate prepared by the sodium hydroxide method (table 78). It is unfortunate that the results of Saxholm (tables 30, 31, 32, 33 and 34), those of Lind (table 35) and those of experiment 7 (table 65) do not provide the evidence required for a final assessment of the efficiency of the pancreatin-desogen method. In the light of the considerations set out above on p. 192 of this thesis it is to be expected that the decision not to test a method critically might be regretted. This is particularly the case with my examination of Saxholm's method because all the methods judged to be more worthy of critical examination than Saxholm's method proved to be less efficient than the sodium hydroxide method - the sodium hydroxide-teepol method (experiment 9, tables 67 and 68), Nassau's swab method (experiment 12, tables 78 and 79), the laryngeal swab method (experiment 13, tables 80 and 81) and a modification of the method of Patterson et al. (experiment 14, tables 86 and 87).

Experiment 11. This trial of thefluid media EV and PF (see p. 74 of this thesis) suggests that fluid media are of little value for the isolation of tubercle bacilli from sputum. I

have suggested on p. 156 of this thesis that the main reason for the poor performance of these media is that they failed to control the growth of contaminants; even when the inoculum was treated with sodium hydroxide the PF medium failed to control contamination. The failure of these media merits further consideration.

There are 3 reasons why control of contamination was poor. First, even if only a few of the organisms other than tubercle bacilli were able to grow in spite of the bacteriostatic agents incorporated in the EV and PF medium, they will soon outgrow the tubercle bacillus in a fluid medium and produce conditions in which it is unable to multiply. Second, the bacteriostatic agents and antibiotics used for the EV and PF media were selected without exhaustive preliminary trial (see p. 144 and p. 158 of this thesis); they were selected because the properties of each agent seemed appropriate to a medium of this kind; moreover, they were used in combination without any tests of their mutual compatibility and without tests to exclude a possible bacteriostatic action of the mixture on tubercle bacilli. Third, although antibiotics and dyes are frequently used for the suppression of unwanted organisms in routine laboratory practice - for example, in the isolation of Haemophilus pertussis from sputum or the isolation of intestinal

pathogens from faeces - growth of contaminants has usually to be suppressed for only a few days; on the other hand, on primary isolation, macroscopic growth of tubercle bacilli will not be obtained in less than 10 days and cultures must be incubated for at least 6 weeks before being discarded as negative. The long incubation period required for the isolation of tubercle bacilli produces 2 additional difficulties in the control of contamination: first, contaminants whose growth is not completely suppressed have a good opportunity to spoil the medium and second, the action of unstable bacteriostatic agents like antibiotics decreases during the incubation period. Further, in specimens containing few tubercle bacilli, contaminants are usually present in overwhelming numbers and this increases the chance that the inoculum will contain organisms which are naturally resistant to antibiotics.

Experiment 14. This experiment illustrates the need for patience in examining methods of isolating tubercle bacilli from sputum. I wished to examine the method described by Patterson et al. (see p. 33 of this thesis) - a method which has no advantage in simplicity or safety over the sodium hydroxide method because it involves exactly the same use of the centrifuge as the sodium hydroxide method. Nevertheless the results of Patterson et al. suggest that

the method is more efficient than the sodium hydroxide method (table 86). A preliminary trial of a simple method of concentrating tubercle bacilli by means of barium sulphate sedimentation (see p.169 of this thesis) gave promising results with homogenates prepared by the sodium hydroxide method (tables 83 and 84). Therefore I modified the method of Patterson et al. by substituting barium sulphate sedimentation for centrifugation. My modification of the method of Patterson et al. proved less efficient than the sodium hydroxide method. Therefore, it must be concluded either that the relatively poor results yielded by the modification of the method of Patterson et al. are attributable to the simplification or that the poor results are attributable to the decontamination procedure described by Patterson et al. and that the results described by Patterson et al. are misleading. Figures 8 and 9 suggest that barium sulphate sedimentation is not a reliable method of concentrating tubercle bacilli from homogenates of sputum; fig. 8 shows that culture by my modification of the method of Patterson et al. is as efficient as the sodium hydroxide method whereas fig. 9 shows that modification of the method of Patterson et al. is less efficient than the sodium hydroxide method. Moreover, table 87 shows that 3 specimens yielded a positive

result only by the modification of the method of Patterson et al. whereas 14 specimens yielded a positive result only by the sodium hydroxide method; in the light of the appearances shown in figs. 8 and 9 this suggests very strongly that barium sulphate sedimentation is occasionally able to concentrate tubercle bacilli from a sputum homogenate with great efficiency but that the concentrating action of this procedure differs from specimen to specimen. Experiment 14 is therefore presented as evidence that barium sulphate sedimentation is not an acceptable alternative to centrifugation; further examination of the original method described by Patterson et al. is required in order to establish whether this method is more efficient than the sodium hydroxide method for specimens containing few tubercle bacilli.

SUGGESTIONS FOR FUTURE WORK ON METHODS OF ISOLATING TUBERCLE BACILLI FROM SPUTUM

The most disappointing feature of my experimental work is that it has failed to establish a safe, simple and efficient method of isolating tubercle bacilli from sputum - a method which could be applied routinely to all specimens. The results of my experiments (tables 70, 72, 78, 80 and 86) show that, for specimens likely to contain only small numbers of tubercle bacilli, the most practicable ways of avoiding centrifugation in the isolation of tubercle bacilli from sputum are less efficient than the sodium hydroxide method. I believe that the most certain way of avoiding centrifugation without impairing efficiency is to culture the whole untreated specimen without preliminary decontamination. The results obtained in a preliminary study of direct culture of sputum in the fluid medium EV (see p. 156 and table 72 of this thesis) indicate that the main problems requiring further study are 1) control of contamination and 2) provision of a medium with suitable nutrient qualities.

Control of contamination.

Two main methods are generally used for the separation of one particular micro-organism from a mixture of micro-organisms. One method, dating from the time of

Koch, is physical and relies on simple separation of the organisms on the surface or in the depth of a solid medium; the other method is biological and depends on the fact that the bacteriostatic or bactericidal action of certain substances - for example, dyes and antibiotics - differs from one micro-organism to another. These well-established methods should be applied to further studies in the isolation of tubercle bacilli from untreated specimens of sputum.

Physical control of contamination. Agar is the basis of most solid media; silica gels have also been used. For the primary isolation of tubercle bacilli from untreated sputum, surface cultures on media solidified with agar or silica are impracticable because of the large surface area required to ensure satisfactory separation of tubercle bacilli from other micro-organisms with an inoculum of 2 to 10 ml. of sputum. Culture of tubercle bacilli in the deep agar or silica gel media is also impracticable because it is difficult to recover colonies from deep cultures for the purposes of microscopic examination and subculture and because anaerobiosis in the depth of the medium will prevent the growth of tubercle bacilli. Methyl cellulose has the following properties which might make it suitable for the isolation of tubercle bacilli in the depth of a thin layer of solid medium. First, it is a relatively inert chemical; second, media containing methyl cellulose are fluid at room temperature and solid at 37°C.

Consequently, it may be possible 1) to separate tubercle bacilli from contaminants by maintaining the media at 37°C in the solid state until the tubercle bacilli have had an opportunity to multiply and 2) to liquify the medium by chilling and recover a representative sample of the culture by means of a pasteur pipette fitted with a rubber teat.

Biological control of contamination. The studies reported in experiment 6 were unsatisfactory (see p. 194 of this thesis). Therefore, it would be worth while to examine thoroughly dyes, antibiotics, quaternary ammonium compounds and other selective inhibitors of bacterial growth; a very wide range of these agents has become available in recent years and it is highly probable that an agent or combination of agents may be found which, in conjunction with the physical measures described above, may retard the growth of normal sputum flora and permit growth of tubercle bacilli. The following points in the study of these agents require emphasis. First, tests with laboratory strains of tubercle bacilli should be used only as a rough guide for the selection of suitable agents. Second, the properties of combinations of suitable agents should not be assumed to be the sum of the properties of each agent in the combination; it is most important to ensure that the combination of agents does not result in a mixture which inhibits the growth

of tubercle bacilli or fails to give the expected control of contamination. Third, because cultures for the isolation of tubercle bacilli require a long incubation period, the stability of the agents should be investigated in the medium in which they are to be used. Fourth, the influence of the medium and of sputum on the bacteriostatic properties of the agents should be borne in mind; for example, it is well known that the bacteriostatic power of substances like silver nitrate, malachite green, and etnyl violet are impaired by the presence of protein.

Provision of a suitable medium for the isolation of tubercle bacilli from sputum.

Löwenstein-Jensen medium is unsuitable for the isolation of tubercle bacilli from untreated sputum mainly because only the surface is available for culture (see above p. 202 of this thesis); further, the egg yolk in this medium interferes with the action of bacteriostatic agents. Moreover, because egg yolk is the main constituent of this medium, its efficiency is subject to uncontrollable variation in the quality of eggs. Nevertheless, it is doubtful whether any of the media which do not contain egg yolk are as efficient as Löwenstein-Jensen medium. Recently Yamane (1957) has described a medium in which a derivative of egg yolk has been used. This derivative does not contain nitrogen or phosphorous;

it melts at 36°C; it has a characteristic and easily recognisable crystalline structure; it is not destroyed by autoclaving at 120°C for 20 minutes. Yamane claims that an agar medium incorporating this fraction of egg yolk is a better nutrient for the H37Rv strain of tubercle bacilli than a modification of Löwenstein-Jensen medium. The heat stability and transparency of media containing the egg yolk fraction are properties which make the Yamane type of medium ideal for depth culture of tubercle bacilli. Experiments should therefore be made in order to establish whether media containing this fraction of egg yolk are equal to or superior to Lowenstein-Jensen medium for the isolation of tubercle bacilli from sputum.

Clearly the experimental approach described above is a formidable undertaking. Nevertheless, I believe that it offers the best prospect of evolving a simple, safe and efficient method of isolating tubercle bacilli from sputum.

Possible criticisms of this approach to the isolation of tubercle bacilli are 1) that microscopy would probably be necessary in order to establish whether or not a culture was positive and 2) that decontaminating agents might still have to be used on a positive culture in order to obtain a pure culture of tubercle bacilli. Although these criticisms have an important bearing on simplicity and safety, they must be

viewed in the light of routine laboratory practice. One of the main objections to the sodium hydroxide method and the other standard methods is that the initial handling of specimens is complicated and time-consuming; it is not easy to regulate the numbers of specimens submitted daily to the laboratory and at present laboratory workers are often required to deal with numbers of specimens of sputum which are in excess of the number which can be handled safely. In my opinion, the disadvantages of direct culture - the need for microscopy and decontamination after a preliminary period of incubation - should not be over-emphasised for the following reasons. First, the initial handling of specimens is reduced to a minimum and therefore relatively little time, care and skill would be required for this work. Second, the dangers attached to microscopic examination and decontamination after incubation of the primary cultures would probably be smaller than those at present encountered in the performance of sensitivity tests; for example, the swab method of Nassau (see p.107 of this thesis) would be a safe and simple procedure for the decontamination of positive cultures. Third, even if the examination of cultures proved to be time-consuming, this work can readily be regulated so that the number of examinations made on a given day is within the capacity of the laboratory staff. In other words, methods used at present are neither safe nor

simple and the pressure of work on the operator is governed mainly by a factor which is not readily controlled by the laboratory staff - the number of specimens submitted on a given day; the procedure which might be established by the work outlined above may not have a great over-all advantage over existing methods in simplicity or safety but, because the initial treatment of specimens would be exceedingly simple, the most time-consuming and dangerous laboratory operations arise at a point where the pressure of work can be foreseen and regulated by the laboratory staff so that proper attention may be given to safety precautions.

SUMMARY

This thesis contains the following chapters.

1. Introduction. This chapter sketches briefly the reasons why methods of isolating tubercle bacilli from sputum should be re-examined. The reasons are: a) laboratory procedures play an important part in the diagnosis, treatment and control of tuberculosis; b) present methods of isolating tubercle bacilli from sputum are unsatisfactory - most methods are time-consuming, and many are dangerous to the operator; c) although much has been written about the efficiency of methods of isolating tubercle bacilli from sputum, little attention has been paid to other important criteria by which methods should be judged - simplicity and safety.

2. Literature. I have reviewed the literature concerning methods of isolating tubercle bacilli from sputum or other specimens obtained from patients suffering from pulmonary tuberculosis. In addition I have assessed the simplicity and safety of laboratory procedures commonly used for the diagnosis, treatment and control of tuberculosis. The following conclusions are reached.

a) Significant differences between methods of isolating tubercle bacilli from sputum can be demonstrated by comparing the results obtained by the methods from series of specimens

of sputum likely to contain only small numbers of tubercle bacilli.

b) Trials with series of specimens of sputum establish only the relative efficiency of methods and therefore fail to indicate whether it is worthwhile to search for more efficient methods.

c) The results of studies made by means of viable counting procedures do not always agree with the results of studies made with series of specimens of sputum. Information obtained by means of a viable counting procedure - a relatively artificial method - must therefore be supplemented by studies with series of specimens of sputum - a procedure which is closely related to routine laboratory practice.

d) Because they do not involve centrifugation, Saxholm's pancreatin-desogen method and Nassau's swab method are the most attractive of the recently described procedures for preparing sputum for the culture of tubercle bacilli.

e) Microcultural methods yield earlier results than standard methods. They are unsuitable for routine application because they require additional laboratory space and greater numbers of skilled technicians.

f) Laryngeal swab culture is the simplest method of isolating tubercle bacilli from patients who are unable to

produce sputum.

g) Methods of isolating tubercle bacilli from sputum and methods of determining the sensitivity of tubercle bacilli to antituberculous drugs expose the operator to the danger of infection with tubercle bacilli. This danger can be reduced in two ways: first, by a search for methods which reduce the likelihood of liberating tubercle bacilli in the form of aerosols and second, by preventing the inhalation of aerosols containing tubercle bacilli. The second way of reducing the danger of laboratory infection depends mainly on the provision of ventilated safety-hoods for the manipulation of tuberculous material.

3. Experiments. This chapter contains an account of my own experiments. The following points are established.
a) Methods of isolating tubercle bacilli from sputum should be assessed by comparison with a standard method in series of specimens of sputum. For this purpose the most suitable standard method is the sodium hydroxide method.
b) The selection of specimens of sputum is of vital importance in comparing methods of isolating tubercle bacilli from sputum. I evolved a critical procedure for assessing the efficiency of methods; 3 replicate specimens are examined from each of 40 to 50 patients whose sputum is likely to contain very few tubercle bacilli. This procedure can be applied with very small resources of technical assistance and will show clearly

any important difference in the efficiency of two methods.

c) Viable counting procedures are of little value in the assessment of methods of isolating tubercle bacilli from sputum.

d) When specimens containing very few viable tubercle bacilli are cultured by the sodium hydroxide method concentration by centrifugation may not be omitted without a serious loss in the efficiency of the method. Preliminary examination of Saxholm's pancreatin-desogen method - a method which does not involve centrifugation - suggests that this method would prove less efficient than the sodium hydroxide method. Other methods which avoid centrifugation proved significantly less efficient than the sodium hydroxide method.

e) Nassau's swab method is highly efficient for specimens known from microscopy to contain acid-fast bacilli. This method is safe and simple; it does not involve centrifugation. Therefore, in preparing sputum for culture, the risk of laboratory infection with tubercle bacilli may be reduced in the following way. Specimens positive by microscopy - those from which the most dangerous aerosols are produced by shaking and centrifugation - should be cultured by Nassau's swab method; only specimens negative by microscopy - those from which very small numbers of tubercle bacilli are likely to be released in the form of an aerosol by shaking or

centrifugation - should be cultured by the more efficient but more time-consuming and dangerous methods like the sodium hydroxide method.

4. Discussion. This chapter includes a critical evaluation of the experimental procedures used for this thesis, a discussion of the facts established by the experiments, a review of experiments considered to be unsatisfactory and suggestions for future work on methods of isolating tubercle bacilli from sputum. It is claimed that the procedures adopted in my experiments were the correct ones for the circumstances under which the work was accomplished and that failure to apply these or similar procedures lessens the value of many of the studies discussed in the review of the literature. The unsatisfactory nature of some of my experiments is discussed in the light of the well-known difficulties involved in work with the tubercle bacillus. It is suggested that future work on the isolation of tubercle bacilli should be directed towards a simplification of the initial laboratory treatment of specimens; an experimental approach to this problem is described.

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A CRITICAL SURVEY AND EXPERIMENTAL STUDY OF METHODS OF ISOLATING TUBERCLE BACILLI FROM SPUTUM

THESIS FOR THE DEGREE OF DOCTOR OF MEDICINE PRESENTED BY

A. J. O'HEA, M.B., Ch.B.

VOLUME II: Tables and Illustrations

Table 1

Sodium hydroxide versus antiformin for the isolation of tubercle bacilli from sputum. Figures from Lurie (1923). Each specimen cultured by both methods. Gentian violet-egg medium

Method	Negative by microscopy	Positive by microscopy	Total
Sodium hydroxide			
Number of examinations	20	34	54
Number positive	6	14	20
Per cent. culture positive	30	41.2	37
Antiformin x			
Number of examinations	20	34	54
Number positive	1	2	3
Per cent. culture positive	5	5.9	5.6

x Sputum was homogenised with an equal volume of 20 per cent. antiformin

Table 2.

Efficiency of methods of cultivating tubercle bacilli from sputum. Results of a trial conducted by the Public Health Laboratory Service (1952). Presentation of results in order of efficiency

Method	Negative by direct microscopy	Positive by direct microscopy	Total
4 per cent. sodium hydroxide.			
Number of examinations	1,243	361	1,604
Number positive	356	311	667
Per cent. culture positive	28.6	86.1	41.6
3 per cent. sulphuric acid.			
Number of examinations	1,222	349	1,571
Number positive	267	287	554
Per cent. culture positive	21.8	82.2	35.3
Acid-iron-peroxide.			
Number of examinations	1,280	333	1,613
Number positive	250	268	518
Per cent. culture positive	19.5	80.5	32.1
Trisodium phosphate.			
Number of examinations	1,199	347	1,546
Number positive	202	264	466
Per cent. culture positive	16.8	76.1	30.1

Contamination rates (Sodium hydroxide = 4.9 per cent; Sulphuric acid = 4.0 per cent; Acid-iron-peroxide = 5.2 per cent; Trisodium phosphate = 7.6 per cent) of all slopes inoculated

Table 3

Efficiency of methods of cultivating tubercle bacilli from sputum. Figures taken from Baker (1951) and presented in order of efficiency for specimens negative for acid-fast bacilli by direct microscopy

Method	Negative by direct microscopy	Positive by direct microscopy	Total
Sodium hydroxide			
Number of examinations	96	24	120
Number positive	26	24	50
Per cent. culture positive	28.1	100	41.7
Trisodium phosphate			
Number of examinations	99	22	121
Number positive	16	22	38
Per cent. culture positive	16.2	100	31.4
Sodium hydroxide modified by Hanks et al.			
Number of examinations	87	33	120
Number positive	14	27	41
Per cent. culture positive	16.1	81.1	34.2
Oxalic acid			
Number of examinations	78	16	94
Number positive	10	10	20
Per cent. culture positive	12.8	62.5	21.2
Acid-iron-peroxide			
Number of examinations	91	16	107
Number positive	5	15	20
Per cent. culture positive	5.5	93.7	18.7

Contamination rates:- Figures not given in original paper

Table 4

Sodium hydroxide versus trisodium phosphate for the isolation of tubercle bacilli from sputum. Figures from Mullany (1950). Each specimen divided equally between the two methods. Dorset egg medium

Method	Concentrate negative for acid-fast bacilli	Concentrate positive for acid-fast bacilli	Total
Sodium hydroxide			
Number of examinations	217	46	263
Number positive	86	32	118
Per cent. culture positive	39.6	69.6	44.9
Trisodium phosphate			
Number of examinations	209 x	54 x	263
Number positive	53	26	79
Per cent. culture positive	25.4	48.2	30

Contamination rate:- Sodium hydroxide — 8 per cent. of all slopes inoculated

Trisodium phosphate — 30.4 per cent. of all slopes inoculated

x These figures differ from those given for the sodium hydroxide method because more specimens were positive after concentration by trisodium phosphate than after sodium hydroxide

Table 5

Sodium hydroxide versus trisodium phosphate for the isolation of tubercle bacilli from sputum. Figures from Byham (1950). A sample of each of 89 specimens examined by each method. No facts available about microscopic examination of specimens. Oleic acid-albumin agar medium

Method	Percentage of positive results
Sodium hydroxide	89.6
Trisodium phosphate	73.9

Contamination rate:-	Sodium hydroxide	Trisodium phosphate
	6.4 per cent. of all slopes inoculated	20.2 per cent. of all slopes inoculated

Table 6

Sodium hydroxide versus trisodium phosphate for the isolation of tubercle bacilli from sputum. Presumably a different series of specimens was used for each method but this is not clearly stated in the original paper; no information concerning medium. Final concentration of trisodium phosphate - 3.25 per cent. of the anhydrous salt. From Starkey and Aubert (1950)

Source	Method	No. of specimens	Contaminated per cent.	Positive culture negative microscopy per cent.
General hospital wards	NaOH	2,600	6	5
	Na_3PO_4	2,340	3.3	4 [x]
Sanatorium wards	NaOH	1,436	2.5	8.7
	Na_3PO_4	1,256	1.7	10.2
Specimens received by mail from outpatients	NaOH	895	14	3
	Na_3PO_4	1,274	7.2	7.2

[x] The 5% figure for NaOH is probably too high. Lack of staff prevented careful scrutiny of smears and therefore specimens microscopically positive by the standards later adopted may have been included as microscopically negative in the sodium hydroxide figures

Table 7

Sodium hydroxide versus trisodium phosphate for the isolation of tubercle bacilli from sputum (Gifford et al., 1951). Two tubes of Petragnani medium (modified by McNabb 1936) and 2 of Löwenstein Jensen medium inoculated by each method. Each specimen examined by both methods

Method	Negative by microscopy	Positive by microscopy	Total
Sodium hydroxide Number of examinations	x . . .	. . .	2,368
Number positive	. . .	. . .	500
Per cent. culture positive	. . .	. . .	21.1
Trisodium phosphate Number of examinations	. ..	. . .	2,368
Number positive	. . .	. . .	521
Per cent. culture positive	. . .	. . .	22

Contamination rate:- Sodium hydroxide: 1.03 per cent. of all specimens investigated

Trisodium phosphate: 3.85 per cent. of all specimens investigated

Microscopy: Approximately 12.85 per cent. of all specimens examined were positive for acid-fast bacilli

x Figures not given in original paper

Table 8

Trisodium phosphate versus sodium hydroxide for the isolation of tubercle bacilli from sputum. Two samples of sputum collected from each patient: 1) a 24 hr. collection into 50 ml. of 5 per cent. (annydrous) trisodium phosphate; 2) a second 24 hr. collection in the succeeding 24 hrs. into an empty vessel. Specimens in trisodium phosphate shaken, centrifuged, and deposit used for inoculation; specimens without trisodium phosphate treated with 4% sodium hydroxide, centrifuged and deposit used for inoculation. From Pelzer et al., (1954)

Method	Negative by microscopy	Positive by microscopy	Total
Sodium hydroxide			
Number of examinations	...[x]	...	591
Number positive	...	...	217
Per cent. culture positive	...	...	36.7
Trisodium phosphate			
Number of examinations	...	...	591
Number positive	...	...	225
Per cent. culture positive	...	...	38.1

Contamination rate:-	Sodium hydroxide	Trisodium phosphate
	2.4 per cent. of all specimens investigated	0.5 per cent. of all specimens investigated

x Figures not given in original paper

Table 9

Sulphuric acid versus oxalic acid for the isolation of tubercle bacilli from sputum. From Corper and Uyei (1930). Both methods applied to each of 16 specimens of sputum known to contain acid-fast bacilli; 5 tubes of glycerol-potato medium inoculated by each method.

Method	Percentage of tubes giving positive results	Percentage of tubes contaminated
6 per cent. sulphuric acid	78	28
9.5 per cent, oxalic acid	86	15
7 per cent. oxalic acid	85	14
5 per cent. oxalic acid	88	15
2 per cent. oxalic acid	66	45

Table 10

Oxalic acid versus acid-iron-peroxide for the isolation of tubercle bacilli from sputum. From Collins (1952). All specimens from patients in whom three previous specimens were negative by microscopy for acid-fast bacilli. Of 2,171 specimens, 541 examined by both methods, 656 by oxalic acid method only and 833 by acid-iron-peroxide method only. Lowenstein-Jensen medium (3 slopes) and Dorset medium (1 slope) inoculated by each method

Method	All specimens
Oxalic acid	
Number of examinations	1,197
Number positive	183
Per cent. culture positive	15.3
Acid-iron-peroxide	
Number of examinations	974
Number positive	112
Per cent. culture positive	11.5

Contamination:-	Oxalic acid	Acid-iron-peroxide
	28.7 per cent. of all slopes inoculated	32.1 per cent. of all slopes inoculated

Table 11

Sodium hydroxide versus acid-iron-peroxide (hydrogen peroxide used in 10 volume strength) for the isolation of tubercle bacilli from sputum. From Anderson et al. (1953). Each specimens cultured by both methods on Löwenstein-Jensen medium

Method	Negative on direct examination	Positive on direct examination	Total
Sodium hydroxide			
Number of examinations	61	39	100
Number positive	9	20	29
Per cent. culture positive	14.8	51.5	29
Acid-iron-peroxide			
Number of examinations	61	39	100
Number positive	7	24	31
Per cent. culture positive	11.5	61.5	31

Contamination rate:- Figures not given in original paper

Table 12

Sodium hydroxide versus acid-iron peroxide (hydrogen peroxide used at 10 volume strength) for the isolation of tubercle bacilli from sputum. From Macfarlane et al. (1955). Each specimen cultured by both methods on Löwenstein-Jensen medium

Method	Negative on direct examination	Positive on direct examination	Total
Sodium hydroxide			
Number of examinations	100	100	200
Number positive	. . .[x]	. . .	115
Per cent. culture positive	. . .	. . .	57
Acid-iron-peroxide			
Number of examinations	100	100	200
Number positive	. . .	. . .	69
Per cent. culture positive	. . .	. . .	34.5

[x] Figures not given in original paper

Table 13

Sodium hydroxide versus acid-iron-peroxide (hydrogen peroxide used at 1 volume strength) for the isolation of tubercle bacilli from sputum. From Macfarlane et al. (1955). Each specimen cultured by both methods. Löwenstein-Jensen medium

Method	Negative on direct examination	Positive on direct examination	Total
Sodium hydroxide			
Number of examinations	86	114	200
Number positive	. . .[x]	. . .	128
Per cent. culture positive	. . .	. . .	67
Acid-iron-peroxide			
Number of examinations	86	114	200
Number positive	. . .	. . .	80
Per cent. culture positive	. . .	. . .	42

[x] Figures not given in original paper

Table 14

Sodium hydroxide versus sulphuric acid for the isolation of tubercle bacilli from sputum. From Corper and Uyei (1927). Both methods applied to each of 6 specimens of sputum known to contain acid-fast bacilli. Five tubes of glycerol-potato medium inoculated by each method from each specimen

Method	Percentage of tubes giving positive results	Percentage of tubes contaminated
6 per cent. sulphuric acid for 30 minutes	66	5
2 per cent. sodium hydroxide for 30 minutes	60	11

Table 15

Sodium hydroxide versus sulphuric acid for the isolation of tubercle bacilli from sputum and other specimens. Figures from Gernez-Rieux et al. (1949). Two series of specimens, one series treated by each method. Tween-albumin fluid medium

Method	Negative by microscopy	Positive by[x] microscopy	Total
Sodium hydroxide			
Number of examinations	[¤] . . .	. . .	261
Number positive	. . .	. . .	166
Per cent. culture positive	. . .	. . .	63.6
Sulphuric acid ‡			
Number of examinations	. . .	. . .	140
Number positive	. . .	. . .	73
Per cent. culture positive	. . .	. . .	52.1

¤ Figures not given in original paper

x The two series together contain 401 specimens of which 162 were positive by microscopy

‡ 15 per cent. sulphuric acid

Table 16

Sodium hydroxide versus sulphuric acid for the isolation of tubercle bacilli from sputum (Madsen 1950). Each specimen treated by both methods

Method	Negative by microscopy	Positive by microscopy	Total
Sodium hydroxide			
Number of examinations	... x	...	2,516
Number positive	...	...	476
Per cent. culture positive	...	...	18.9
Sulphuric acid			
Number of examinations	...	...	2,516
Number positive	...	...	429
Per cent. culture positive	...	...	17.1

Contamination: Figures for sputum are not given in the original paper but of 10,308 slopes inoculated by each method from different kinds of specimens including the above sputa, 16.9 per cent. were contaminated after sodium hydroxide treatment and 14.6 per cent. after sulphuric acid

x Figures not given in original paper

Table 17

Trisodium phosphate versus oxalic acid for the isolation of tubercle bacilli from sputum. Figures from Corper and Stoner (1946). Each specimen treated by both methods; each specimen negative by microscopy and by guinea pig inoculation. Egg yolk medium of Corper and Conn (1933a)

Method	Results
Trisodium phosphate - 24 hrs at 37°C	
Number of examinations	18
Number positive	3
Per cent. positive	16.7
Oxalic acid - 1 hr at 37°C	
Number of examinations	18
Number positive	0
Per cent. positive	0

Table 18

Trisodium phosphate versus oxalic acid for the cultivation of tubercle bacilli from sputum. Figures from Van Vranken (1947). Each method applied separately to 1,000 consecutive specimens of sputum. Egg yolk medium of Corper and Cohn (1933a)

Method	Negative by direct microscopy	Positive by direct microscopy	Total
Trisodium phosphate			
Number of examinations	887	113	1,000
Number positive	64	63	127
Per cent. positive cultures	7.2	55.8	12.7
Oxalic acid			
Number of examinations	884	116	1,000
Number positive	32	76	108
Per cent. positive cultures	3.6	65.5	10.8

Contamination rate:- Figures not given in original paper

Table 19

Trisodium phosphate versus hydrochloric acid for the isolation of tubercle bacilli from sputum. Figures from Beattie (1949). Each specimen treated by both methods. Petragnani medium

Method	Negative by microscopy	Positive by microscopy	Total
Trisodium phosphate			
Number of examinations	74	171	245
Number positive	. . .[x]	. . .	158
Per cent. culture positive	. . .	. . .	64.5
Hydrochloric acid (McNabb 1936)			
Number of examinations	74	171	245
Number positive	. . .	. . .	171
Per cent. culture positive	. . .	. . .	69.8

Contamination rate:- Figures not given in original paper

x Figures not given in original paper

Table 20

The effect of decontaminating agents on aqueous suspensions of tubercle bacilli. Numeral indicates time in weeks before growth appeared; exponential indicates number of tubes positive of 3 inoculated when less than 3 tubes were positive. Results from Corper and Stoner (1946)

Time of exposure at 37°C	Saline			±3% NaOH			±5% Oxalic acid			±10% Na3PO4		
	Numbers of tubercle bacilli treated (mgm. per ml.)											
	ǂ1	10^{-3}	10^{-6}	1	10^{-3}	10^{-6}	1	10^{-3}	10^{-6}	1	10^{-3}	10^{-6}
0	2	2	3	2	2	5^1	2	2	4	2	2	3
2 hrs	2	2	3	2	3	0†	2	3	6^1	2	2	3
4 hrs	2	2	3	2	3	0	2	3	0	2	2	3
1 day	2	2	3	2	3	0	3	0	0	2	2	3
4 days	2	2	5^2	2	4	0	0	0	0	2	2	4
2 weeks	2	0	0	4	0	0	0	0	0	2	4	6

x ǂ Concentrations before mixing equal volumes of reagent and bacillary suspension

† no growth

Table 21

The effect of decontaminating agents on an aqueous suspension containing 10-5 mgm. of tubercle bacilli. Colony count of tubercle bacilli at different time-intervals. Results from Spendlove et al. (1949)

Exposure time	Decontaminating agent					
	ǂ5 per cent. Na_3PO_4		ǂ2 per cent. NaOH		ǂ2.5 per cent. H_2SO_4	
	Count	Per cent. survival	Count	Per cent. survival	Count	Per cent. survival
x Control	582	100	506	100	478	100
40 min	307	52	235	46	200	41.8
24 hrs	286	49.2	40	8	0	0
72 hrs	262	44.9	15	3	0	0

ǂ Final concentration

x Medium inoculated immediately after mixing bacillary suspension with neutralised decontaminating agent

Table 22

Colony counts of tubercle bacilli at different time-intervals from mixtures of pooled sputum and decontaminating agents. Figures from Spendlove et al. (1949)

Exposure time	x 2% NaOH	x 5% Na_3PO_4	x 2.5% H_2SO	x 2.5% oxalic acid
20 min	U C	U C	U C	C
40 min	U	U	U	U
2 hrs	U	U	1,000	U
24 hrs	580	1,000	0	0
72 hrs	0	60	0	0

x = Final concentration

U = uncountable

C = contaminated.

Table 23

The effect of decontaminating agents on a suspension of tubercle bacilli in albumin water. Figures from Gray et al.. (1954)

Agent and final concentration	Time of exposure	Number of viable organisms x 10^6 per ml.	Percentage of organisms surviving treatment
None	30 min	180	100
2 per cent. NaOH	30 min	23	12.8
5 per cent. Na_3PO_4	24 hrs	22.5	12.5
3 per cent. H_2SO_4	30 min	3.5	1.9
2.5 per cent. oxalic acid	30 min	1.2	0.7
Acid-iron-peroxide	30 min	0.054	<0.1

Table 24

The effect of decontaminating agents on tubercle bacilli suspended in purulent non-tuberculous sputum.

A: (Sputum diluted for counting after treatment. Original mixture contained 11 x 10^5 viable organisms

B: (Sputum diluted before treatment and dilutions treated. Original mixture contained 72 x 10^5 viable organisms per ml

Results from Gray et al. (1954)

	Agent and concentration	Time of exposure	Number of viable organisms x 10^5 per ml.	Percentage of organisms surviving treatment
A	2 per cent. NaOH	30 min.	2	18.2
	5 per cent. Na_3PO_4	24 hrs.	C[x]	...
	2.5 per cent. oxalic acid	30 min.	0.7	6.4
	3 per cent. H_2SO_4	30 min.	0	0
	Acid-iron-peroxide	30 min.	0	0
B	2 per cent. NaOH	30 min.	11	15.3
	5 per cent. Na_3PO_4	24 hrs.	C	...
	2.5 per cent. Oxalic acid	30 min.	1.4	1.9
	3 per cent. H_2SO_4	30 min.	0.3	0.4
	Acid-iron-peroxide	30 min.	0.06	0.1

[x] C = contamination of counting medium

Table 25

The effect of decontaminating agents on tuberculous sputum. Sputum diluted for counting after treatment. Figures from Gray et al. (1954)

Agent and final concentration	Time of exposure	Number of viable organisms at 10^{-3} dilution
2 per cent. NaOH	30 min	6
5 per cent. Na_3PO_4	24 hrs	8
3.5 per cent. Na_3PO_4	24 hrs	10
2.5 per cent. oxalic acid	30 min	3
3 per cent. H_2SO_4	30 min	0
Acid-iron-peroxide	30 min	0

Table 26

The isolation of tubercle bacilli from sputum with Teepol (Browning et al. 1953). Löwenstein-Jensen medium. Duration of exposure to Teepol 6 hrs (one third of the specimens) or 24 hrs (the remainder of the specimens)

Homogenisation with Teepol	Negative by microscopy	Positive by microscopy	Total
Number of examinations	101	41	142
Number positive	8	40	48
Per cent. culture positive	8	97.6	33.8

Table 27

Sodium hydroxide versus Teepol for the isolation of tubercle bacilli from sputum (Tison and Loze, 1954). Each specimen cultured by both methods. Four Löwenstein-Jensen slopes inoculated be each method for each specimen

Method	From specimens negative by microscopy	From specimens positive by microscopy	Total
Sodium hydroxide			
Number of tubes of medium inoculated	. . . [x]	. . .	220
Number positive	. . .	. . .	91
Per cent. tubes yielding positive culture	. . .	. . .	45.9
Teepol			
Number of tubes of medium inoculated	. . .	. . .	220
Number positive	. . .	. . .	87
Per cent. tubes yielding positive culture	. . .	. . .	39.6

Contamination rate:-

Sodium hydroxide	Teepol
3.2 per cent. of all slopes inoculated	4.1 per cent. of all slopes inoculated

x Figures not given in original paper

Table 28

Sodium hydroxide versus sodium hydroxide-teepol for the isolation of tubercle bacilli from sputum. Results from Tison (1954). Each specimen cultured by both methods. Four Löwenstein-Jensen slopes inoculated by each method for each specimen

Method	Result
Sodium hydroxide	
Number of tubes of medium inoculated	384
Number positive	16
Per cent. positive	4.2
Sodium hydroxide-teepol	
Number of tubes of medium inoculated	384
Number positive	27
Per cent. positive	7

Contamination:- Figures not given in original paper

Table 29

Sodium hydroxide versus sodium hydroxide-teepol for the isolation of tubercle bacilli from sputum. Results from Tison and Audrin 1955. Each specimen cultured by both methods. Four Löwenstein-Jensen slopes inoculated by each method for each specimen

Method	Negative by direct microscopy	Positive by direct microscopy	Total
Sodium hydroxide			
Number of examinations	...[x]	...	400
Number positive	...	...	75
Per cent. positive	...	...	18.8
Sodium hydroxide-teepol			
Number of examinations	...	...	400
Number positive	...	...	109
Per cent. positive	...	...	27.3

Contamination:	Sodium hydroxide	Sodium hydroxide-teepol
Number of specimens in which all 4 slopes were contaminated	3 (7.3 per cent.)	0
Number of specimens in which one to three of the slopes were contaminated	39 (9.8 per cent.)	25 (6.3 per cent.)

x Figures not given in original paper

Table 30

Pancreatin-desogen (4 hours) versus sodium hydroxide for the isolation of tubercle bacilli from sputum. Each specimen cultured by both methods. From each specimen, 4 Löwenstein-Jensen slopes inoculated by each method. Results from Saxnolm (1954)

Method	Negative by direct microscopy	Positive by direct microscopy	Total
Sodium hydroxide			
Number of examinations	. . . [x]	. . .	661
Number positive	. . .	. . .	128
Per cent. culture positive	. . .	. . .	19.3
Pancreatin-desogen			
Number of examinations	. . .	. . .	661
Number positive	. . .	. . .	149
Per cent. culture positive	. . .	. . .	22.5

Contamination:-	Sodium hydroxide	Pancreatin-desogen
	15 per cent. of all slopes inoculated	17.7 per cent. of all slopes inoculated

x Figures not given in original paper

Table 31

Pancreatin-desogen (24 hours) versus sodium hydroxide for the isolation of tubercle bacilli from sputum. Each specimen cultured by both methods. Four Löwenstein-Jensen slopes inoculated by each method from each specimen. Results from Saxholm (1954)

Method	Negative by direct microscopy	Positive by direct microscopy	Total
Sodium hydroxide			
Number of examinations	. . . x	. . .	230
Number positive	. . .	. . .	37
Per cent. culture positive	. . .	. . .	16.1
Pancreatin-desogen			
Number of examinations	. . .	. . .	230
Number positive	. . .	. . .	57
Per cent. culture positive	. . .	. . .	24.8

Contamination rate: Sodium hydroxide — 17.3 per cent. of all slopes inoculated

Pancreatin-desogen — 13.7 per cent. of all slopes inoculated

x Figures not given in original paper

Table 32

Pancreatin-desogen (1 per cent. Desogen, 19 or 44 hours' treatment) versus sodium hydroxide for the isolation of tubercle bacilli from sputum. Each specimen cultured by both methods. Löwenstein-Jensen medium. Results from Saxholm (1955)

Method	Negative by microscopy	Positive by microscopy	Total
Sodium hydroxide			
Number of examinations	x . . .	. . .	2,575
Number positive	. . .	. . .	351
Per cent. culture positive	. . .	. . .	13.6
Pancreatin-desogen			
Number of examinations	. . .	. . .	2,575
Number positive	. . .	. . .	348
Per cent. culture positive	. . .	. . .	13.1

Contamination rate:-

Sodium hydroxide	Pancreatin-desogen
1.2 per cent. of all specimens examined	10.5 per cent. of all specimens examined

x Figures not given in original paper

Table 33

Pancreatin-desogen (1.5 per cent. Desogen, 19 or 44 hours' treatment) versus sodium hydroxide for the isolation of tubercle bacilli from sputum. Each specimen treated by both methods. Löwenstein-Jensen medium. Results from Saxholm (1955)

Method	Negative by microscopy	Positive by microscopy	Total
Sodium hydroxide			
Number of examinations	x . . .	. . .	1,243
Number positive	. . .	. . .	157
Per cent. culture positive	. . .	. . .	12.6
Pancreatin-desogen			
Number of examinations	. . .	. . .	1,243
Number positive	. . .	. . .	170
Per cent. culture positive	. . .	. . .	13.7

Contamination rate:	Sodium hydroxide	Pancreatin-desogen
	1.1 per cent. of all specimens examined	2.4 per cent. of all specimens examined

x Figures not given in original paper

Table 84

Comparison of sodium hydroxide, pancreatin - 1 per cent. desogen and pancreatin - 1 per cent. benzalkonium chloride for the isolation of tubercle bacilli from sputum. Treatment with pancreatin methods for 4 or for 24 hrs. Löwenstein Jensen medium. Results from Saxnolm (1955)

Method	Negative by microscopy	Positive by microscopy	Total
Sodium hydroxide			
Number of examinations	...[x]	...	572
Number positive	...	...	91
Per cent. culture positive	...	...	15.9
Pancreatin-desogen			
Number of examinations	...	...	572
Number positive	...	...	116
Per cent. culture positive	...	...	20.3
Pancreatin-benzalkonium chloride			
Number of examinations	...	...	572
Number positive	...	...	66
Per cent. culture positive	...	...	11.5

Contamination rates:- Benzalkonium chloride is approximately as efficient as desogen; figures not given

x Figures not given in original paper

Table 35

Sodium hydroxide versus pancreatin - 1 per cent. Desogen (24 hours' treatment) for the isolation of tubercle bacilli from sputum (Lind, 1955). Each specimen cultured by both methods. Sediment from each method inoculated on four Löwenstein-Jensen slopes

Method	Negative by direct microscopy	Positive by direct microscopy	Total
Sodium hydroxide			
Number of examinations	x . . .	. . .	463
Number positive	. . .	. . .	152
Per cent. culture positive	. . .	. . .	32.8
Pancreatin-desogen			
Number of examinations	. . .	. . .	463
Number positive	. . .	. . .	137
Per cent. culture positive	. . .	. . .	29.6

Contamination rates:-	Sodium hydroxide	Pancreatin-desogen
	9.7 per cent. of all slopes inoculated	20.1 per cent. of all slopes inoculated

x Figures not given in original paper

Table 36

Sodium hydroxide versus trisodium phosphate plus benzalkonium chloride for the isolation of tubercle bacilli from sputum. (Patterson et al., 1955). Ninety-five specimens examined. Each specimen treated by both methods. Petragnani medium

Method	Negative by microscopy	Positive by microscopy	Total
Sodium hydroxide			
Number of examinations	30.5 per cent.	69.5 per cent.	95
Number positive	...x	...	...
Per cent. culture positive	...	...	50.51
Trisodium phosphate-benzalkonium chloride			
Number of examinations	ŧ 32.5 per cent.	ŧ 67.5 per cent.	95
Number positive	...	...	...
Per cent. culture positive	...	...	60

Contamination rates:- Sodium hydroxide — 13.7 per cent. of all slopes inoculated

Trisodium phosphate-benzalkonium chloride — 2.1 per cent. of all slopes inoculated

x Figures not given in original paper

ŧ These figures differ from those given for the sodium hydroxide method because more specimens were positive by microscopy after concentration by sodium hydroxide than after trisodium phosphate-benzalkonium chloride

Table 37

Trisodium phosphate versus trisodium phosphate plus benzalkonium chloride for the isolation of tubercle from sputum (Patterson et al., 1956). One hundred and fifty-three specimens examined, each specimen treated by both methods. Petragnani medium

Method	Negative by microscopy	Positive by microscopy	Total
Trisodium phosphate			
Number of examinations	83 per cent.	17 per cent.	153
Number positive	...x	...	...
Per cent. culture positive	...	...	21.6
Trisodium phosphate-benzalkonium chloride			
Number of examinations	83 per cent.	17 per cent.	153
Number positive	...	...	...
Per cent. culture positive	...	...	82.7

Contamination rates:- Trisodium phosphate 7.2 per cent. of all slopes inoculated; Trisodium phosphate benzalkonium chloride 0.7 per cent. of all slopes inoculated

x Figures not given in original paper

Table 38

Slide culture versus culture by the sodium hydroxide method on Löwenstein-Jensen medium for the isolation of tubercle bacilli from sputum. All specimens treated by both methods. Results from Oeding, (1951)

Method	Negative by direct microscopy	Positive by direct microscopy	Total
Sodium hydroxide			
Number of examinations	169	31	200
Number positive	44	31	75
Per cent. culture positive	26	100	37.5
Slide culture			
Number of examinations	169	31	200
Number positive	15	31	46
Per cent. culture positive	8.9	100	23

Contamination rates:- Figures not given in original paper

Table 39

Slide culture versus culture by the sodium hydroxide method on Löwenstein-Jensen medium for the isolation of tubercle bacilli from sputum. Each specimen treated by both methods. Results from Hesselberg and Oeding (1952)

Method	Negative by microscopy	Positive by microscopy	Total
Sodium hydroxide			
Number of examinations	1,934	219	2,153
Number positive	343	211	554
Per cent. culture positive	17.7	96.3	25.7
Slide culture			
Number of examinations	1,934	219	2,153
Number positive	156	211	367
Per cent. culture positive	8.1	96.3	17

Contamination rates:- Figures not given in original paper

Table 40

Sodium hydroxide versus micro-culture for the isolation of tubercle bacilli from sputum. All specimens treated by both methods. Data from Berry and Lowry (1950)

Method	Negative by microscopy	Positive by microscopy	Total
Sodium hydroxide			
Number of examinations	937	36	973
Number positive	44	27	71
Per cent. culture positive	4.7	75	7.6
Micro-culture			
Number of examinations	937	36	973
Number positive	19	29	48
Per cent. culture positive	2	80.6	4.9

Contamination rates:- Sodium hydroxide: 12 per cent. of all specimens examined

Micro-culture: 9 per cent. of all specimens examined

Table 41

Slide culture versus trisodium phosphate homogenisation for the culture of tubercle bacilli from sputum. (Simpson and Reed, 1955). Each specimen cultured by both methods

Method	Concentrate microscopically negative	Concentrate microscopically positive	Total
Slide culture			
Number of examinations	2,018	230	2,248
Number positive	56	213	269
Per cent. culture positive	2.8	92.6	12
Trisodium phosphate			
Number of examinations	2,018	230	2,248
Number positive	57	161	218
Per cent. culture positive	2.8	70	9.7

Contamination rates:- Figures not given in original paper

Table 42

Micro-culture versus the sodium hydroxide method for the isolation of tubercle bacilli from sputum. All specimens negative for acid-fast bacilli by microscopy. Each specimen cultured by both methods. Results from Chu (1955)

Method	Results	Mean time for recognition of positive result
Micro-culture		
Number of examinations	56	
Number positive	30	2.5 days
Per cent. culture positive	53.5	
Sodium hydroxide		
Number of examinations	56	
Number positive	23	31.1 days
Per cent. culture positive	41.7	

Contamination rates:- Figures not given in original paper

Table 43

Micro-culture (filter paper strips on solid medium for 10 days) versus sodium hydroxide[x] for the isolation of tubercle bacilli from sputum. Löwenstein-Jensen medium. Each specimen cultured by both methods. Results from Hoyt et al. (1954)

Method	Positive by microscopy	Negative by microscopy	Total
Sodium hydroxide			
Number of examinations	... ǂ	...	470
Number positive	...	...	78
Per cent. culture positive	...	...	16.6
Micro-culture			
Number of examinations	...	...	470
Number positive	...	...	58
Per cent. culture positive	...	...	12.3

x Final concentration for most specimens was 0.75 per cent.

ǂ Figures not given in original paper

Table 44

Laryngeal swab versus gastric lavage for the isolation of tubercle bacilli in cases of pulmonary tuberculosis a) hospital patients and (b) out-patients (Forbes et al., 1948). Each patient examined by both methods. Two laryngeal swabs taken at each investigation. Löwenstein-Jensen medium

	Laryngeal swab	Gastric lavage
a) In-patients		
Number of examinations	100	100
Number positive	32	42
Per cent. culture positive	32	42
b) Out-patients		
Number of examinations	101	101
Number positive	13	11
Per cent. culture positive	12.9	10.9

Table 45

Gastric lavage versus laryngeal swab for the isolation of tubercle bacilli from patients. All patients investigated by both methods. Two laryngeal swabs taken from each patient. Löwenstein-Jensen and Peizer-Schecter medium inoculated from each specimen. Results from Chaves, Peizer & Widelock (1953)

Method	Result
Gastric lavage	
Number of examinations	1,418
Number positive	187
Per cent. culture positive	13.2
Laryngeal swab	
Number of examinations	1,418
Number positive	135
Per cent. culture positive	9.6

Table 46

Laryngeal swabs versus gastric lavage for the isolation of tubercle bacilli from patients. Each patient investigated by both methods on one to three occasions. Swabs treated by alkali. Results from Tonge and Hughes, (1956)

Method	Patients	Specimens
Gastric lavage		
Number investigated	465	1,305
Number positive	130	268
Per cent. culture positive	28	20.5
Laryngeal swab		
Number investigated	465	1,305
Number positive	54	99
Per cent. culture positive	11.6	7.6

Table 47

Laryngeal swab versus sputum culture for the isolation of tubercle bacilli in cases of pulmonary tuberculosis. Majority of sputum specimens negative by microscopy. Each patient examined by both methods. Two laryngeal swabs taken at each investigation. Löwenstein-Jensen medium. Results from Forbes et al. (1948)

Method	Result
Sputum culture	
Number of examinations	96
Number positive	28
Per cent. culture positive	29.2
Laryngeal swab culture	
Number of examinations	96
Number positive	16
Per cent. culture positive	16.7

Table 48

The efficiency of different types of specimens in isolating tubercle bacilli from 107 patients. Each patient on one or more occasions contributed four specimens viz. sputum, gastric contents, laryngeal swab, urine. Results from Hata et al., (1950)

Specimen	No. of specimens	Per cent. positive
Sputum homogenate not concentrated	130	35.4
Sputum homogenate concentrated by centrifugation	130	37.7
Gastric washings	130	43.9
Laryngeal swab	130	16.9
Urine	130	2.3

Table 49

Tracheal lavage versus gastric lavage for the isolation of tubercle bacilli in cases of pulmonary tuberculosis (Wardrip et al., 1949). Investigation of 199 patients negative by direct methods

Method	Result
Tracheal lavage	
Number of examinations	199
Number positive	111
Per cent. culture positive	55.8
Gastric lavage	
Number of examinations	86 x
Number positive	24
Per cent. culture positive	27.9

x All of these specimens were from the group of 111 patients who yielded positive findings with tracheal lavage. Gastric lavage was never positive in a patient in whom tracheal lavage was negative

Table 50

Tracheal lavage versus gastric lavage for the isolation of tubercle bacilli from patients. Data from Deakins and Barber, 1953. All patients investigated by both methods on same day or by one method one day and alternative method on the following day

Method	Result
Gastric lavage	
Number of examinations	486
Number positive	129
Per cent. culture positive	26.5
Tracheal lavage	
Number of examinations	486
Number positive	112
Per cent. culture positive	23

Table 51

Comparison of tracheo-bronchial lavage, gastric lavage and laryngeal swabbing for the isolation of tubercle bacilli from patients with minimal tuberculosis (Lees et al., 1955). Each method applied to 144 patients

No. of cases	Method	No. positive
144	Any or all	35 (24 per cent.)
144	Tracheo-bronchial lavage	26 (18 per cent.)
144	Gastric lavage	14 (10 per cent.)
144	Laryngeal swabbing	8 (6 per cent.)

Table 52

Comparison of the rates of infection of laboratory workers with tubercle bacilli with the expected rates computed from the general population (Reid, 1957)

Occupation	Observed Rates		Expected Rates	
	Male	Female	Male	Female
Pathologist	12	4	4	0.5
Technician:-				
Chief or Senior	6	0	3	0
Trained	10	2	5	2
Junior	14	5	3	5
Student	11	12	2	3
Post-mortem attendant	10	-	1	-

Table 53

The distribution of 1,342 laboratory infections according to the type of work performed by the victim. Of these 1,342 infections, 51 per cent. were certainly acquired in the laboratory, 40 per cent. probably acquired in the laboratory and 9 per cent. possibly acquired in the laboratory. Figures from Sulkin and Pike (1951)

Occupation	Diagnostic	Research	Production of biological material	Class work	Combination of activities
Number	455	308	25	29	525
Per cent. of total	33.9	23	1.9	2.2	39.1

Table 54

Effect of mechanical shaking on the efficiency of the oxalic acid method for isolating tubercle bacilli from sputum. Figures from Collins, (1951). Two series of specimens, one series homogenised by mechanical shaking, the other by hand shaking. Each specimen allocated to one method only. Löwenstein-Jensen medium

Method	Results
Mechanical shaking	
Number of examinations	388
Number positive	102
Per cent. culture positive	26.3
Hand shaking	
Number of examinations	379
Number positive	87
Per cent. culture positive	22.9

Table 55

Table 55

Efficiency of methods of isolating tubercle bacilli from sputum. Numbers of viable organisms surviving each treatment. Equal portions of each specimen (2 ml.) treated by each method. Sediments obtained by each method (swab method excepted) suspended in 2 ml. of Proskauer and Beck medium and counting dilutions made from these suspensions. Control counting dilutions made in Proskauer and Beck medium and in Proskauer and Beck medium containing 100 units per ml. of penicillin from an untreated portion of specimen. Each of 3 swabs charged with 0.02 ml. of each of the higher dilutions of untreated specimen and decontaminated with 5 per cent. oxalic acid. Each count is the mean of 3 replicates

Table 55

Sputum no.	Dilution at which count was possible	No. of viable organisms in 0.02 ml. of given dilution						
		Control		Sodium hydroxide	Trisodium phosphate	Sulphuric acid	Acid-iron-peroxide	Swab (Oxalic acid)
		[x]No P	P					
1	4×10^{-2}	‡C	50	51	52	48	42	11
2	4×10^{-3}	C	37	6.5	5.8	31	28	3.7
3	4×10^{-1}	C	C	47	23	66	52	10
4	4×10^{-3}	21	24	4.2	15	16	10	0.4
5	4×10^{-4}	C	14	1.9	1.4	4.1	4.3	0.02
6	4×10^{-4}	38	43	5	3.3	31	2.4	...
7	4×10	C	11	4.4	2.9	13	5.4	4

[x]No P without penicillin in the diluting fluid

P with penicillin in the diluting fluid

‡C contaminated

Table 56

Efficiency of methods of isolating tubercle bacilli from sputum. Numbers of viable organisms found in the supernatant fluids and in the corresponding sediments obtained by centrifuging homogenates of sputum. Each count is the mean of 3 replicates

Sputum number	Count made from	No. of viable organisms in 0.02 ml. of dilution							
		Sodium hydroxide		Trisodium phosphate		Sulphuric acid		Acid-iron-peroxide	
		10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}
5	Sediment	U[x]	19	U	14	U	41	U	43
	Supernatant	7	0	27	2	9	0	2	0
		10^{-2}	10^{-3}	10^{-2}	10^{-3}	†10^{-3}	10^{-4}	10^{-2}	10^{-3}
6	Sediment	U	50	U	33	U	31	U	24
	Supernatant	25	2	6	1	6	1	25	1

x U uncountable

† Note that this count was made from a lower dilution than the others in this table

Table 57

Numbers of viable tubercle bacilli recovered from sputum a) untreated, b) homogenised with pancreatin, c) homogenised with sodium hydroxide, d) homogenised with sulphuric acid. Counting dilutions for a) and b) made in Proskauer and Beck medium containing 1: 200000 proflavine. Counting dilutions for c) and d) made in Proskauer and Beck medium without proflavine. All counts are means of 5 replicates

Sputum no.	Dilution	Numbers of viable tubercle bacilli in 0.02 ml. of dilution			
		Not homogenised	Pancreatin	Sodium hydroxide	Sulphuric acid
1	10^{-2}	35	40	30	26
	10^{-3}	7	6	5	2
2	10^{-1}	31	33	22	28
	10^{-2}	8	6	4	3
3	10^{-3}	35	39	27	32
	10^{-4}	7	8	5	3

Table 58

Table 58

Effect of neutralisation of sputum homogenates made with sodium hydroxide or sulphuric acid. Numbers of viable organisms isolated from portions of homogenate diluted without neutralisation, diluted after exact neutralisation or after adding excess of neutralising agent. Alkaline homogenates neutralised with sulphuric acid; acid homogenates neutralised with sodium hydroxide. All portions of each sputum brought to the same volume before counting. Each count is the mean of 3 replicates

Table 58

Sputum number	Treatment of homogenate before counting	Sodium hydroxide homogenate			Sulphuric acid homogenate		
		No. of organisms in 0.02 ml. of dilution			No. of organisms in 0.02 ml. of dilution		
		10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}
1	Not neutralised	U[x]	30	4	U	17	1
	Exactly neutralised	U	30	3	U	21	1
	Excess of neutraliser added	18	5	0	U	26	3
2	Not neutralised	32	4	0	32	6	1
	Exactly neutralised	32	4	1	25	3	0
	Excess of neutraliser added	23	3	0	25	4	0
3	Not neutralised	30	5	1	39	9	1
	Exactly neutralised	30	2	0	43	8	2
	Excess of neutraliser added	28	3	2	41	3	1

U uncountable

Table 59

Effect of neutralisation of sputum homogenised with 4 per cent. sodium hydroxide or 3 per cent. (V/V) hydrochloric acid. Numbers of viable organisms isolated from portions of homogenate diluted without neutralisation, diluted after exact neutralisation, diluted after adding excess of neutralising agent. Alkaline homogenates neutralised with 10 per cent. (V/V) hydrochloric acid; acid homogenates neutralised with 4 per cent. sodium hydroxide. All portions of each sputum brought to the same volume before diluting for counting. Each count is the mean of 3 replicates

Sputum number	Treatment of homogenate before counting	Sodium hydroxide			Hydrochloric acid		
		No. of organisms in 0.02 ml. of dilution			No. of organisms in 0.02 ml. of dilution		
		10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
4	Not neutralised	36	7	0	35	7	1
	Exactly neutralised	42	9	1	40	6	1
	Excess of neutraliser added	36	4	0	41	13	2
5	Not neutralised	U[x]	U	40	U	U	40
	Exactly neutralised	U	U	40	U	U	40
	Excess of neutraliser added	U	U	15	U	U	39
6	Not neutralised	U	21	2	U	41	5
	Exactly neutralised	U	29	5	U	41	3
	Excess of neutraliser added	20	2	0	U	46	8

x U uncountable

Table 60

Effect of washing, neutralisation and centrifugation on the number of colonies isolated from sputum homogenised with sodium hydroxide. Each count is the mean of 3 replicates. All counts made from the same volume of material

Treatment	Not neutralised			Neutralised		
	No. of colonies in 0.02 ml. of dilution			No. of colonies in 0.02 ml. of dilution		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Homogenate	U^{x}	>40‡	15	U	37	3
Sediment obtained by centrifugation	U	>40	15	U	33	8
Supernatant obtained by centrifugation	U	23	2	>1	0	0
Sediment obtained after resuspending and centrifuging first sediment						

U^{x} = uncountable

‡ = the number of colonies was too great to be counted accurately

Table 61

Table 61

Comparison of results obtained by the sodium hydroxide method with those obtained by the sulphuric acid method in 11 patients who yielded a positive result by either or both methods in 1 or more of 3 replicate specimens. Two Löwenstein-Jensen slopes inoculated by each method. All specimens negative for acid-fast bacilli by direct microscopy

Table 61

Patient number	Result of culture [x]					
	First replicate specimen		Second replicate specimen		Third replicate specimen	
	Sodium hydroxide	Sulphuric acid	Sodium hydroxide	Sulphuric acid	Sodium hydroxide	Sulphuric acid
1	− −	− −	+ −	+ +	− −	+ +
2	− C	− −	+ −	− −	− −	− −
3	+ +	+ +	+ +	+ +	− −	+ +
4	+ +	− −	− −	− −	− −	− C
5	+ +	+ +	− −	− −	− −	− −
6	+ +	+ C	− −	− −	+ −	+ C
7	+ C	+ +	+ +	+ −	+ C	+ −
8	+ C	+ C	− C	− C	− −	− −
9	+ +	− −	− C	− −	− −	− −
10	+ −	+ −	− −	− −	− −	− −
11	− −	− −	+ +	− −	+ +	+ +
Total no. of patients positive	8	6	5	3	3	5
Cumulative total no. of patients positive	8	6	11	7	11	8

x ++ = tubercle bacilli isolated on both slopes) called positive
+− = tubercle bacilli isolated on one slope; other slope sterile) called positive
+C = tubercle bacilli isolated on one slope; other slope contaminated) called positive

−− = both slopes sterile) called negative
−C = one slope sterile; other slope contaminated) called negative
CC = both slopes contaminated) called negative

Table 62

Comparison of results obtained by the sodium hydroxide method and by the sulphuric acid method in 120 specimens of sputum obtained from 40 patients (3 replicate specimens from each patient). All specimens negative for acid-fast bacilli by direct microscopy

	Number	Per cent.
Specimens examined	120	100
Specimens positive by:-		
Either or both methods	18	15
Both methods	12	10
Sodium hydroxide method	16	13.3
Sulphuric acid method	14	11.7
Sodium hydroxide method only	4	3.3
Sulphuric acid method only	2	1.7

Table 63

Comparison of contamination rates of sodium hydroxide and sulphuric acid methods. Two Lowenstein-Jensen slopes inoculated by each method from each of 120 specimens

Assessment of contamination	Method	
	Sodium hydroxide	Sulphuric acid
Slopes contaminated /slopes inoculated	46/240 (19.2 per cent.)	17/240 (7.1 per cent.)
Specimens yielding no result because of contamination of both slopes/specimens examined	13/120 (10.8 per cent)	2/120 (1.7 per cent)

Table 64

Action of selective bacteriostatic agents on H37Rv strain of tubercle bacilli in medium of Proskauer and Beck with 10 per cent. human serum. Surface culture incubated 10 to 14 days; result expressed as growth in comparison with that obtained in control medium without bacteriostatic agent

Agent	Inhibitory action on tubercle bacilli		
	Complete	Partial	None
	Concentration	Concentration	Concentration
Etnyl violet	1:10,000	. . .	1:100,000
Proflavine	1:10,000	1:100,000	1:250,000
Malachite green	1:10,000	. . .	1:100,000
Oxalic acid	1:200	. . .	1:2,000
Potassium tellurite	1:10,000	1:250,000	1:500,000
Sodium azide	1:10,000	1:25,000	1:50,000
Silver nitrate	1:100,000	. . .	1:250,000
Hibitane	1:100,000	. . .	1:250,000

Table 65

Isolation of tubercle bacilli from specimens of sputum known to contain acid-fast bacilli. Homogenisation and decontamination with combinations of pancreatin with antibacterial agents. Homogenates inoculated on Lowenstein-Jensen medium 1) by means of a loop and 2) by means of a pasteur pipette. Homogenates not centrifuged and not washed

Agent combined with 1 per cent. pancreatin	No. of specimens investigated	Method of inoculation	Result of culture		
			No. positive	No. negative	No. contaminated
Malachite green 0.05 per cent.	10	Loop	1	1	8
		Pipette	1	1	8
Ethyl violet 0.05 per cent.	5	Loop	1	0	4
		Pipette	1	0	4
Proflavine 0.05 per cent.	5	Loop	3	1	1
		Pipette	5	0	0
Hibitane 0.5 per cent.	16	Loop	8	0	8
		Pipette	8	0	8
Desogen 0.5 per cent.	6	Loop	4	2	0
		Pipette	6	0	0
Silver nitrate 0.5 per cent.	4	Loop	0	0	4
		Pipette	0	0	4
Mercuric chloride 0.5 per cent.	4	Loop	0	0	4
		Pipette	0	0	4

Table 66

Comparison of trisodium phosphate with mixtures of trisodium phosphate and Hibitane or proflavine for the isolation of tubercle bacilli from sputum. All homogenates neutralised before inoculation on Löwenstein-Jensen medium

Decontaminating agent	Concentration of trisodium phosphate - anhydrous salt per cent.	No. of positive results obtained from 4 specimens of sputum
Trisodium phosphate	10	4
	5	4
	1	3
Trisodium phosphate plus 0.01 per cent. Hibitane	10	2 x
	5	1 x
	1	4
Trisodium phosphate plus 0.01 per cent. proflavine	10	0
	5	0
	1	0

x Contamination in 1 of the 4 specimens

Table 67

Table 67

Comparison of results obtained by sodium hydroxide method with those obtained by the sodium hydroxide-teepol method in 7 patients who yielded a positive result by either or both methods from 1 or more of 3 replicate specimens. Two Löwenstein-Jensen slopes inoculated by each method. All specimens negative for acid-fast bacilli by direct microscopy

Table 67

Patient number	Result of culture of[x]											
	First replicate specimen				Second replicate specimen				Third replicate specimen			
	Sodium hydroxide method		Sodium hydroxide-teepol method		Sodium hydroxide method		Sodium hydroxide-teepol method		Sodium hydroxide method		Sodium hydroxide-teepol method	
1	C	C	+	C	-	C	-	-	-	-	-	-
2	+	+	+	+	+	+	+	+	+	+	+	+
3	-	-	-	-	-	-	-	C	+	+	-	-
4	+	C	+	+	+	+	+	+	+	+	+	-
5	-	C	-	C	-	-	-	-	+	C	+	C
6	-	-	-	-	+	-	-	-	-	-	-	-
7	+	+	-	-	-	-	-	-	-	-	-	-
Total no. of patients positive	3		3		3		2		4		3	
Cumulative total no. of patients positive	3		3		4		3		6		4	

x See footnote to table 61 for explanation of symbols

Table 68

Comparison of results obtained by the sodium hydroxide method and by the sodium hydroxide-teepol method in 45 specimens of sputum from 15 patients, (each patient contributed 3 replicate specimens). All specimens negative for acid-fast bacilli by direct microscopy

	Number	Per cent.
Specimens examined	45	100
Specimens positive by:-		
Either or both methods	11	24.4
Both methods	7	15.6
Sodium hydroxide method	10	22.2
Sodium hydroxide-teepol method	8	17.8
Sodium hydroxide method only	3	6.7
Sodium hydroxide-teepol method only	1	2.2

Table 69

Comparison of contamination rates of the sodium hydroxide and sodium hydroxide-teepol methods. Two Löwenstein-Jensen slopes inoculated by each method from each of 45 specimens

Assessment of contamination rate	Method	
	Sodium hydroxide	Sodium hydroxide-teepol
Slopes contaminated /slopes inoculated	24/90 (26.7 per cent.)	9/90 (10 per cent.)
Specimens yielding no result because of contamination of both slopes/ specimens examined	7/45 (15.6 per cent)	0/45

Table 70

The value of centrifugation in the isolation of tubercle bacilli from sputum. Comparison of the numbers of positive results obtained from 74 specimens of sputum by the sodium hydroxide when the homogenate is centrifuged with the number obtained when a portion of the homogenate is inoculated without preliminary concentration by centrifugation. Two Löwenstein-Jensen slopes inoculated by each method

	Number	Per cent.
Specimens examined	74	100
Specimens positive:-		
With or without centrifugation	19	25.7
With and without centrifugation	11	14.9
Only with centrifugation	8	10.8
Only without centrifugation	0	

Table 71

Comparison of the contamination rate obtained by the sodium hydroxide method when the homogenate is centrifuged and the rate obtained when a portion of the homogenate is inoculated without preliminary concentration by centrifugation. Two Löwenstein-Jensen slopes inoculated from each of the specimens by each method

Assessment of contamination rate	Method	
	Centrifugation	No centrifugation
Slopes contaminated /slopes inoculated	28/148 (18.9 per cent.)	21/148 (14.2 per cent.)
Specimens yielding no result because of contamination of both slopes/ specimens investigated	5/74 (6.8 per cent.)	5/74 (6.8 per cent.)

Table 72

Comparison of results obtained by the sodium hydroxide method with those obtained by direct culture in E.V. fluid medium in 32 specimens of sputum, each obtained from a different patient. All specimens negative for acid-fast bacilli by direct microscopy, 7 positive only after concentration and 25 negative even after concentration

	All	Positive only after concentration with sodium hydroxide	Negative even after concentration with sodium hydroxide
Number of specimens examined	32	7	25
Positive results obtained by:-			
Either or both methods	11	6	5
Both methods	3	3	0
Sodium hydroxide method	11	6	5
E.V. method	3	3	0
Sodium hydroxide method only	8	3	5
E.V. method only	0	0	0

-Table 73

Comparison of results obtained by the sodium hydroxide method with those obtained by homogenisation with 0.5 per cent. final concentration of sodium hydroxide and subsequent culture of the whole homogenate in P.F. medium. Specimens of sputum obtained from 27 patients - 3 positive for acid-fast bacilli by direct microscopy, 5 positive only after concentration with sodium hydroxide and the remaining 19 negative even after concentration

	Type of Specimen			
	All	Positive by direct microscopy	Positive only after concentration	Negative even after concentration
Number of specimens examined	27	3	5	19
Positive results obtained by:-				
Either or both methods	12	3	5	4
Both methods	3	1	2	0
Sodium hydroxide method	12	3	5	4
P.F. method	3	1	2	0
Sodium hydroxide method only	9	2	3	4
P.F. method only	0	0	0	0

Table 74

Results of culture by the swab method of sputum showing acid-fast bacilli in the direct smear. Two slopes inoculated from each specimen

No. of specimens (out of 96 examined) showing culture results [x]					
++	+-	+c	--	-c	cc
81	4	7	3	0	1
92 positive			4 negative		

x See footnote to table 61 for explanation of symbols.

Contamination rate:-

Slopes contaminated/slopes inoculated	9/192 (4.6 per cent.)
Specimens yielding no result because of contamination/specimens investigated	1/96 (1 per cent.)

Table 75

Comparison of the results of culture by the swab method and by the sodium hydroxide method of sputum showing acid-fast bacilli only after concentration

	Number	Per cent.
Specimens examined	30	100
Specimens positive by:-		
Either or both methods	27	90
Both methods	23	76
Swab method	24	80
Sodium hydroxide method	26	86
Swab method only	1	3.3
Sodium hydroxide method only	3	10

Table 76

Comparison of efficiency of swab and sodium hydroxide methods in specimens of sputum each from a different patient. Sputum not showing acid-fast bacilli even after concentration

	All specimens		Specimens from which 2 uncontaminated slopes were obtained	
	Number	Per cent.	Number	Per cent.
Specimens examined	130	100	89	100
Specimens positive by:-				
Either or both methods	34	26.2	28	31.5
Both methods	19	14.6	17	19.1
Swab method	22	16.9	18	20.2
Sodium hydroxide method	31	23.9	27	30.3
Swab method only	3	2.3	1	1.1
Sodium hydroxide method only	12	9.2	10	11.2

Table 77

Comparison of contamination rates obtained with the sodium hydroxide method and those obtained with Nassau's swab method. Two Löwenstein-Jensen slopes inoculated from each specimens by each method; 130 specimens examined, each from a different patient

Assessment of contamination rate	Method	
	Sodium hydroxide	Swab
Slopes contaminated /slopes inoculated	41/260 (16 per cent.)	21./260 (8 per cent.)
Specimens yielding no result because of contamination of both slopes/ specimens examined	13/130 (10 per cent.)	3/130 (2.3 per cent.)

Table 78

Table 78

Comparison of results obtained by the sodium hydroxide method with those of Nassau's swab method in 19 patients who yielded a positive result by either or both methods in 1 or more of a group of replicate specimens (2 or 3 from each patient). Two Löwenstein-Jensen slopes inoculated by each method. All specimens negative for acid-fast bacilli by direct microscopy

Table 78

Patient number	Result of culture of [x]											
	First replicate specimen				Second replicate specimen				Third replicate specimen			
	Sodium hydroxide method		Swab method		Sodium hydroxide method		Swab method		Sodium hydroxide method		Swab method	
1	−	−	−	−	−	−	C	C	+	+	−	−
2	+	+	+	−	−	−	−	C	...		...	
3	−	−	−	−	+	+	−	−	...		...	
4	+	−	−	−	−	−	−	−	...		...	
5	+	−	C	C	−	−	−	−	...		...	
6	+	+	+	+	+	−	−	−	+	+	+	+
7	−	C	−	−	−	−	−	−	+	−	−	−
8	C	C	−	C	−	−	−	−	C	C	+	−
9	−	−	−	−	+	+	−	−	...		...	
10	+	+	−	−	−	−	−	−	...		...	
11	+	+	+	+	−	−	−	C	+	−	−	−
12	C	C	−	−	+	C	−	C	...		...	
13	+	−	−	−	−	−	−	−	−	C	C	C
14	+	C	+	−	−	C	−	C	...		...	
15	+	+	+	C	+	+	+	C	+	C	+	C
16	+	+	+	+	+	+	+	C	+	+	−	−
17	−	C	−	−	+	−	+	−	+	−	+	−
18	+	+	+	+	+	+	−	−	...		...	
19	−	−	−	−	+	+	−	−	...		...	
Total no. of patients positive	11		7		9		3		7		4	
Cumulative total no. of patients positive	11		7		16		8		17		9	

x See footnote to table 61 for explanation of symbols

Table 79

Comparison of results obtained by the sodium hydroxide method and by Nassau's swab method in 104 specimens obtained from 41 patients (each patient contributed 2 or 3 replicate specimens). All specimens negative for acid-fast bacilli by direct microscopy

	Number	Per cent.
Specimens examined	104	100
Specimens positive by:-		
Either or both methods	28	26.9
Both methods	13	12.5
Sodium hydroxide method	27	25.9
Swab method	14	13.5
Sodium hydroxide method only	14	13.5
Swab method only	1	1

Table 80

Table 80

Comparison of results obtained with the sodium hydroxide method with those obtained by the laryngeal swab method in 13 patients who yielded a positive result by either or both methods in 1 or more of 3 replicate examinations from each patient. Two Löwenstein-Jensen slopes inoculated by each method. All specimens negative for acid-fast bacilli by direct microscopy

Table 80

Patient number	Result of culture of*							
	First replicate examination		Second replicate examination		Third replicate examination			
	Sodium hydroxide method	Laryngeal swab method	Sodium hydroxide method	Laryngeal swab method	Sodium hydroxide method		Laryngeal swab method	
1	+ +	+ −	+ +	− −	− C		− −	
2	C C	− C	+ C	− −	+ +		− −	
3	+ +	− −	+ +	+ −	+ +		+ +	
4	+ +	− −	+ +	− −	+ +		− −	
5	+ +	+ +	+ C	+ −	+ +		+ +	
6	C C	− C	+ +	− −	C C		+ C	
7	− −	+ −	+ +	+ C	− −		+ −	
8	− −	− −	+ −	− −	− −		− −	
9	+ +	+ +	+ +	+ +	+ +		− −	
10	+ +	− −	+ +	+ −	+ +		− −	
11	+ C	+ C	+ +	− −	+ C		+ −	
12	C C	+ C	+ C	C C	− −		+ +	
13	− −	− −	+ +	− −	+ −		− −	
Total no. of patients positive	7	6	13	5	8		6	
Cumulative total no. of patients positive	7	6	13	8	13		9	

* See table 61 for explanation of footnotes

Table 81

Comparison of results obtained by the sodium hydroxide method and by the laryngeal swab method in 138 specimens from 46 patients (each patient contributed 3 replicate specimens). All specimens negative for acid-fast bacilli by direct microscopy

	Number	Per cent.
Specimens examined	138	100
Specimens positive by:-		
Either or both methods	33	23.9
Both methods	12	8.7
Sodium hydroxide method	28	20.3
Laryngeal swab method	17	12.3
Sodium hydroxide method only	16	11.6
Laryngeal swab method only	5	3.6

Table 82

Comparison of the contamination rates of the sodium hydroxide and laryngeal swab methods. Two Löwenstein-Jensen slopes inoculated from each specimen by each method; each method applied to 138 replicate specimens obtained from 46 patients

Assessment of contamination rate	Method	
	Sodium hydroxide	Laryngeal swab
Slopes contaminated /slopes inoculated	62/276 (22.5 per cent.)	24/276 (8.6 per cent.)
Specimens yielding no result because of contamination of both slopes/ specimens examined	17/138 (12.3 per cent.)	2/138 (1.4 per cent.)

Table 83

Table 83

Comparison of the efficiency of centrifugation with that of barium sulphate sedimentation for the concentration of tubercle bacilli from sputum homogenised by the sodium hydroxide method. Two Löwenstein-Jensen slopes inoculated by each method from each specimen. Analysis of the results obtained in 3 replicate specimens from each of 5 patients who yielded a positive result by either or both methods from 1 or more replicate specimens. All specimens negative for acid-fast bacilli by direct microscopy

Table 83

Patient number	x Result of culture of					
	First replicate specimen		Second replicate specimen		Third replicate specimen	
	Centrifugation	Barium sulphate	Centrifugation	Barium sulphate	Centrifugation	Barium sulphate
1	- -	+ C	- -	C -	- -	- -
2	C C	+ -	+ C	+ C	+ +	+ +
3	+ +	+ -	+ +	+ C	+ +	+ +
4	- -	- C	+ +	+ +	- C	- -
5	+ -	- -	- -	C C	- -	- -
Total no. of patients positive	2	3	3	3	2	2
Cumulative total no. of patients positive	2	3	4	4	4	4

x See footnote to table 61 for explanation of symbols

Table 84

Comparison of the efficiency of centrifugation and barium sulphate sedimentation for concentrating tubercle bacilli from sputum homogenised by the sodium hydroxide method. Results of culture in 45 specimens obtained from 15 patients; each specimen examined by both methods; 2 Löwenstein-Jensen slopes inoculated by each method. All specimens negative for acid-fast bacilli by direct microscopy

	Number	Per cent.
Specimens examined	45	100
Specimens positive by:-		
Either or both methods	9	20
Both methods	6	13.3
Centrifugation	7	15.6
Barium sulphate sedimentation	8	17.8
Centrifugation only	1	2.2
Barium sulphate sedimentation only	2	4.4

Table 85

Comparison of contamination rates in the culture of homogenates of sputum concentrated by centrifugation and by barium sulphate sedimentation. Two Löwenstein-Jensen slopes inoculated by each method from each of 45 specimens

Assessment of contamination rate	Method	
	Centrifugation	Barium sulphate
Slopes contaminated /slopes inoculated	19/90 (21.1 per cent.)	19/90 (21.1 per cent.)
Specimens yielding no result because of contamination of both slopes/ specimens examined	5/45 (11.1 per cent.)	2/45 (4.4 per cent.)

Table 86

Table 86

Comparison of the efficiency of centrifugation and barium sulphate sedimentation for concentrating tubercle bacilli from sputum homogenates. Homogenates for centrifugation prepared by the sodium hydroxide method; homogenates for barium sulphate sedimentation prepared by the method of Patterson et al. (1956). Analysis of results in 3 replicate specimens from 18 patients who yielded a positive result by either or both methods in 1 or more replicate specimens. Two Löwenstein-Jensen slopes inoculated by each method. All specimens negative for acid-fast bacilli by direct microscopy

Table 86

Patient number	Result of culture of x											
	First replicate specimen				Second replicate specimen				Third replicate specimen			
	Centrifugation		Barium sulphate		Centrifugation		Barium sulphate		Centrifugation		Barium sulphate	
1	+	+	+	+	+	+	+	+	+	+	+	+
2	C	C	-	-	+	-	-	-	+	-	-	-
3	-	-	C	-	-	-	+	-	-	-	-	-
4	+	+	+	-	+	+	-	-	C	-	-	-
5	+	+	+	+	+	+	+	+	+	+	+	+
6	-	C	+	-	-	-	C	C	-	C	-	-
7	+	+	-	-	+	+	-	-	+	+	-	-
8	+	-	-	-	+	-	-	-	+	C	+	-
9	+	+	-	-	-	-	-	C	-	-	-	-
10	+	+	-	-	+	+	-	-	-	-	-	C
11	+	+	+	-	-	-	-	-	-	-	-	-
12	+	+	+	+	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	C	+	+	C	C
14	+	+	+	+	+	+	+	+	+	+	+	+
15	-	C	-	-	-	-	+	-	-	-	-	-
16	-	-	-	-	-	-	-	-	+	+	-	C
17	+	+	+	+	-	-	-	-	-	-	-	-
18	-	C	-	-	+	-	-	-	-	-	-	C
Total no. of patients positive	12		9		11		7		9		5	
Cumulative total no. of patients positive	12		9		14		11		15		12	

x See footnote to table 61 for explanation of symbols

Table 87

Comparison of the efficiency of centrifugation and barium sulphate sedimentation for concentrating tubercle bacilli from sputum homogenates. Homogenates for centrifugation prepared by the sodium hydroxide method; homogenates for barium sulphate sedimentation prepared by the method of Patterson et al. (1956). Analysis of the results of culture of 156 specimens obtained from 52 patients. Each specimen cultured by both methods. Two Löwenstein-Jensen slopes inoculated by each method. All specimens negative for acid-fast bacilli by direct microscopy

	Number	Per cent.
Specimens examined	156	100
Specimens positive by:-		
Either or both methods	35	22.4
Both methods	18	11.5
Centrifugation	32	20.5
Barium sulphate sedimentation	21	13.5
Centrifugation only	14	9.0
Barium sulphate sedimentation only	3	2.0

Table 88

Comparison of contamination rates in the cultures of sputum homogenates concentrated by centrifugation and by barium sulphate sedimentation. Homogenates for centrifugation prepared by the sodium hydroxide method; homogenates for barium sulphate sedimentation prepared by the method of Patterson et al. (1956). Two Löwenstein-Jensen slopes inoculated by each method from each of 156 specimens

Assessment of contamination rate	Method	
	Centrifugation	Barium sulphate
Slopes contaminated/ slopes inoculated	52/312 (16.7 per cent.)	44/312 (14.1 per cent.)
Specimens yielding no result because of contamination of both slopes/ specimens examined	14/156 (9 per cent.)	9/156 (5.8 per cent.)

Figure 1

Counting viable tubercle bacilli

Bijou bottles with Löwenstein-Jensen medium after an incubation period of 14 days. Note shallow cup-shaped surface of medium. A small amount of condensation moisture is just visible on the necks of the bottles 4th and 5th from the left. Separate colonies are shown on the surface of the bottles 4th and 5th from the left. X 3/4

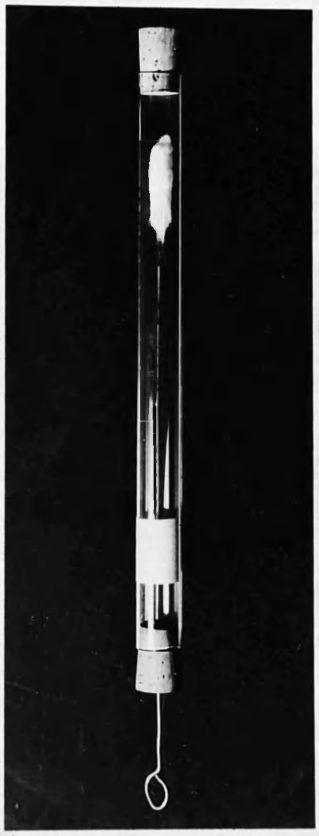
Figure 2

Counting viable tubercle bacilli

The same bottles as shown in fig. 1; the caps have been removed in order to demonstrate the surface growth more clearly. The caps do not require to be removed when making a count in practice. X1

Figure 3

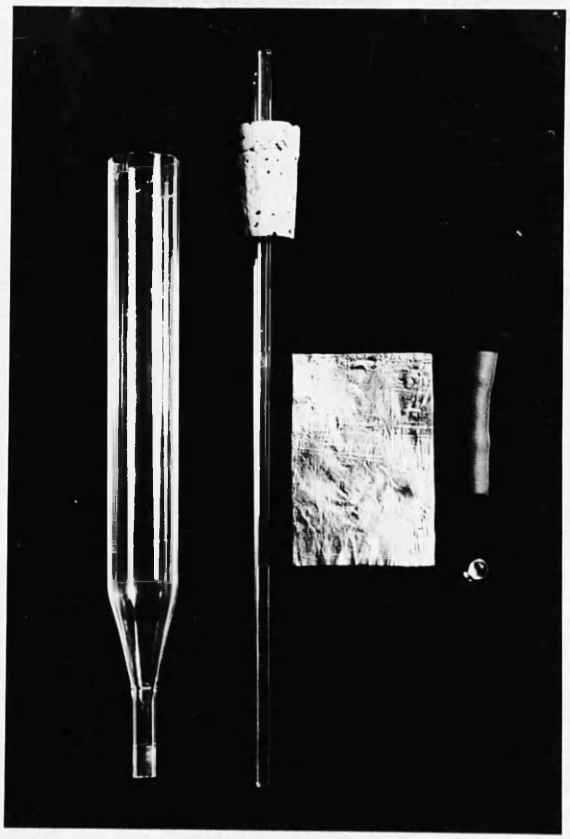
Isolation of tubercle bacilli by means of laryngeal swabs



Laryngeal swab assembled ready for use. Note the generous wrapping of nylon wool. X 1/3

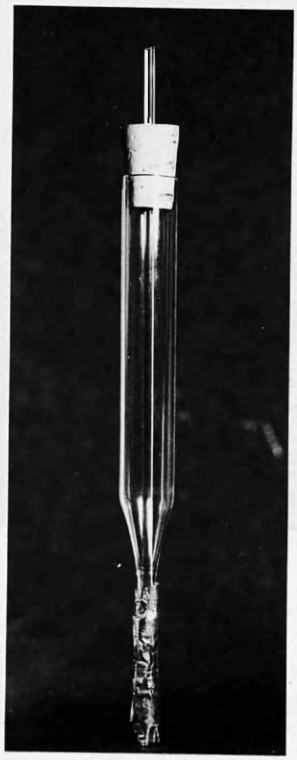
Figure 4

Concentration of homogenates of sputum by means of barium sulphate



Components of sedimentation tube: glass tube, cork stopper with glass rod, lead foil, glass bead, rubber tube. X 1/2

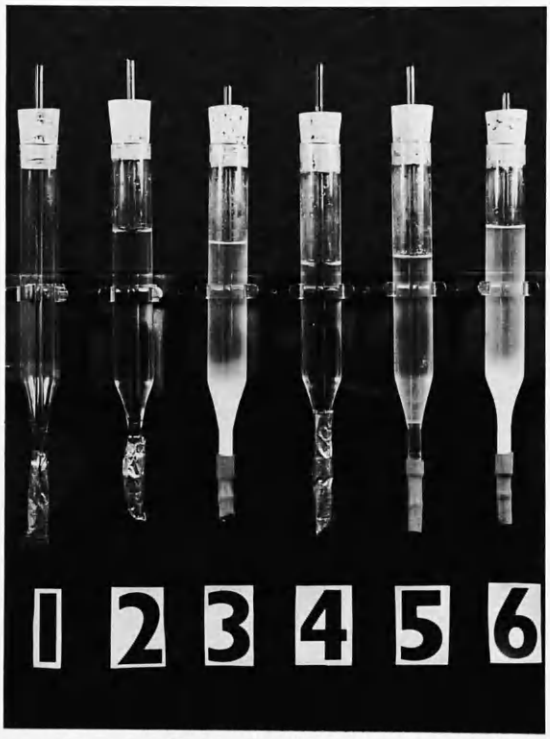
Figure 5



Sedimentation tube assembled from components shown in fig. 4. Note that the stop-cock portion of the apparatus (rubber tube with glass bead) is kept sterile by means of the lead foil wrapping. X 1/2

Figure 6

Concentration of homogenates of sputum by means of barium sulphate



Rack containing sedimentation tubes for concentrating tubercle bacilli by means of barium sulphate. For each homogenate two tubes are required (a and b).
The appearance of the two tubes before commencing the concentration procedure is shown by tubes 1 and 2: tube a (now tube 1) is empty and tube b (now tube 2) contains sterile phosphate buffer at pH7.
Tubes 3 and 4 show the appearance of tubes a and b when sputum homogenate mixed with barium sulphate has been added to tube a (now tube 3) and allowed to stand for 10 minutes. Note that most of the barium sulphate has fallen to the bottom of tube a (now tube 3) and that the lead foil has been removed from the rubber tube so that the sediment may be run into tube b (now tube 4).
Tubes 5 and 6 show the appearances of tubes a and b 10 minutes after the sediment from tube a (now tube 5) has been run into tube b (now tube 6). Note that the lead foil has been removed from tube b (now tube 6) so that the sediment may be run on to the surface of 2 slopes of Löwenstein-Jensen medium. X 1/4

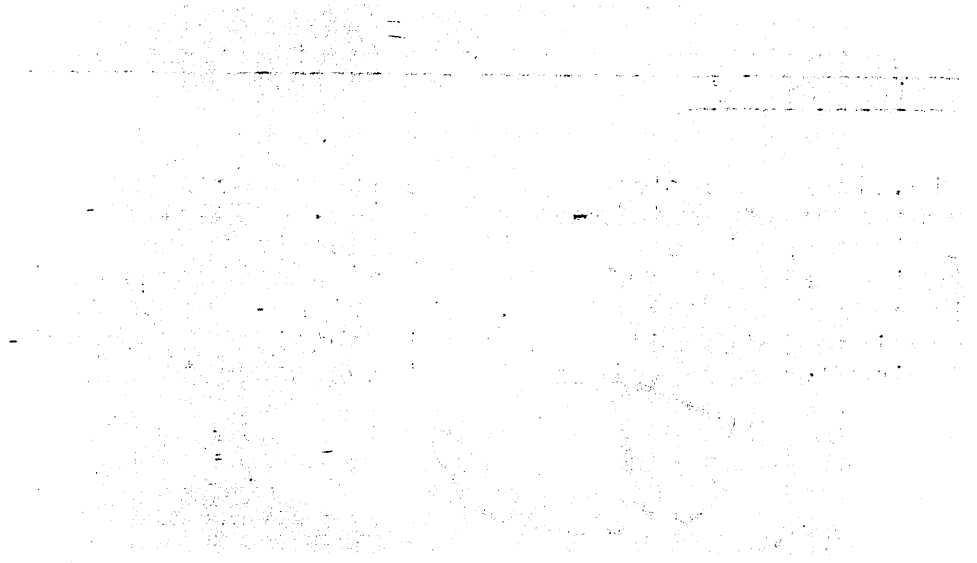


Figure 7

Figure 7A

Concentration of homogenates of sputum by means of barium sulphate

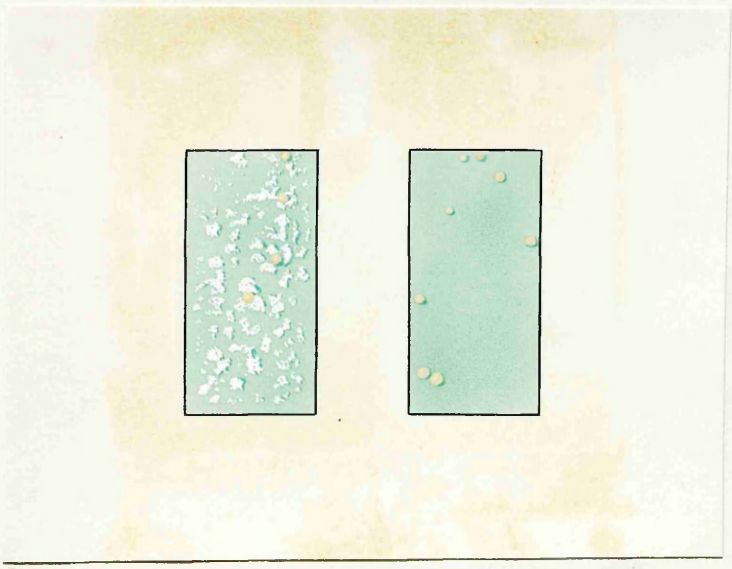
Fig. 7A is a photograph of 2 cultures from the same sputum on Löwenstein-Jensen medium. The left-hand culture was inoculated with a concentrate made with barium sulphate from a portion of a sodium hydroxide homogenate of the sputum. The right-hand culture was inoculated with the deposit obtained by centrifuging the same volume of sputum homogenate

Figure 7B

Fig. 7B is a coloured drawing of the main part of the surface of each of the cultures shown in fig. 7A. Note that approximately the same number of colonies grow on each slope; in this instance there is no striking difference in efficiency between concentration by barium sulphate and concentration by centrifugation. X 5/6

Figure 7A

Figure 7B



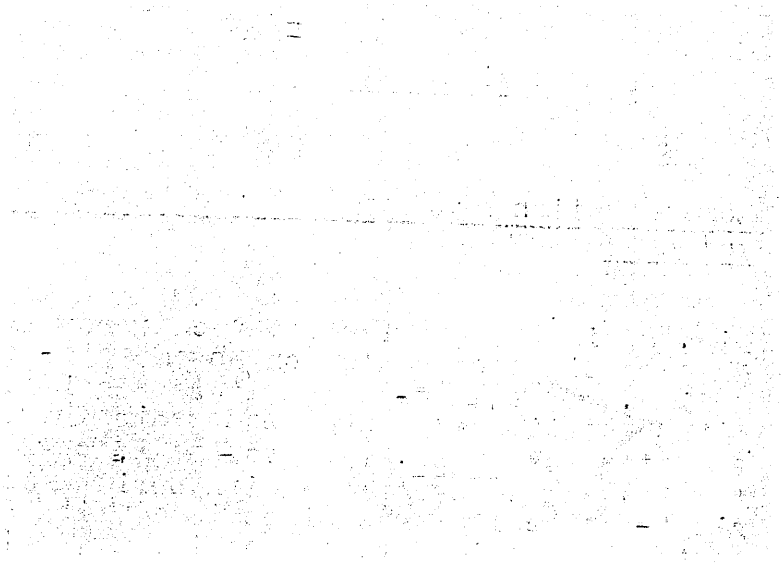


Figure 8

Figure 8A

Concentration of homogenates of sputum of barium sulphate

Fig. 8A is a photograph of 2 cultures from the same specimen of sputum on Löwenstein-Jensen medium. The left-hand culture was inoculated with a portion of sputum homogenised by the method of Patterson et al. (see volume 1, page 116 of this thesis) and concentrated with barium sulphate. The right-hand culture was inoculated with a portion of sputum homogenised by the sodium hydroxide method and concentrated by centrifugation

Figure 8B

Fig. 8B is a coloured drawing of the main part of the surface of the two cultures shown in fig. 8A. Note that the numbers of colonies on both slopes is approximately the same; in this mixture there is no striking difference in efficiency between concentration by barium sulphate and concentration by centrifugation. X 5/6

Figure 8A

Figure 8B

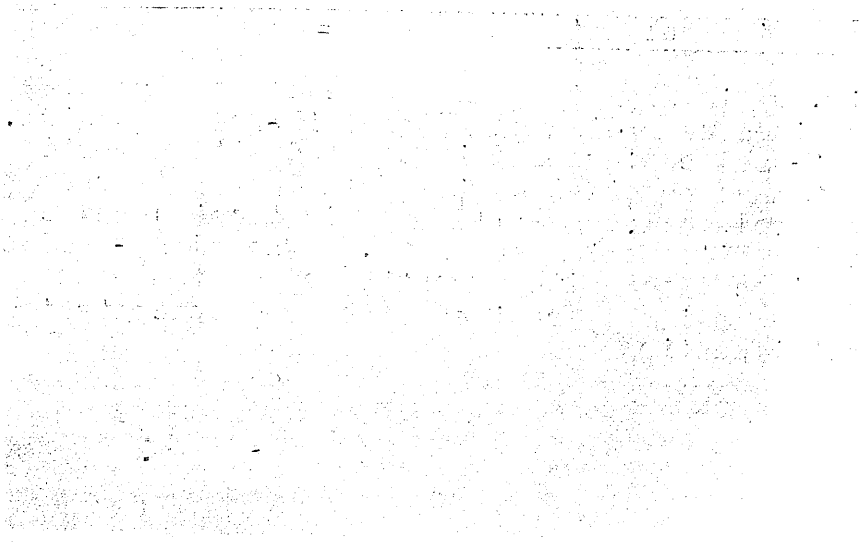


Figure 9

Figure 9A

Concentration of homogenates of sputum by means of barium sulphate

Fig. 9A is a photograph of 2 cultures from the same specimen of sputum on Löwenstein-Jensen medium. The left-hand culture was inoculated with a portion of sputum homogenised by the method of Patterson et al. (see volume 1, page 116 of this thesis) and concentrated with barium sulphate. The right-hand culture was inoculated with a portion of sputum homogenised by the sodium hydroxide method and concentrated by centrifugation

Figure 9B

Fig. 9B is a coloured drawing of the main part of the surface of the two cultures shown in fig. 9A. Note that the left-hand culture from the concentrate prepared by the barium sulphate method yielded less than 20 colonies whereas the right-hand culture inoculated with the centrifuged deposit prepared by the sodium hydroxide method yielded semi-confluent growth of innumerable minute colonies. In this instance concentration by means of barium sulphate is very much less efficient than concentration by centrifugation. X 5/6

Figure 9A

Figure 9B