A GUIDE TO THE COUNTING OF MYCOBACTERIA IN CLINICAL AND EXPERIMENTAL MATERIALS[1, 2]

JOHN H. HANKS, PH.D., B. R. CHATTERJEE, M.B., B.S. AND MICHEL F. LECHAT, M.D.[3]

Johns Hopkins University-Leonard Wood Memorial Leprosy Research Laboratory
The Johns Hopkins School of Hygiene
Baltimore, Maryland

Since *Mycobacterium leprae* is not available in sufficient purity for standardization by weight, spectrophotometry or nitrogen content, bacterial counts are required to determine its concentration in lepromin and in experiments on animal transmission, cell cultures, direct cultivation, etc. When bacterial concentrations in source materials are modest, direct counts often are feasible. When concentrations are high, large clumps and globi require declumping of the bacilli by chloroform and dispersion in a suitable diluent. While the laboriousness of counting is well known, several determinants of reliable counts are not commonly recognized, as indicated in the following paragraphs (A-D).

A. Markedly different, though reliable, distributions of bacterial concentrations are produced by each method of spreading samples on glass slides. After mechanical spreading of measured samples on square or circular areas, the numbers of bacteria per field near the centers of films may exceed those near the perimeters by five to ten times ([5]). Random microscopic sampling of these physically induced variations in bacterial concentrations results in enormous standard errors for the counts from each film. From this it follows that films must be small in order that their entire diameters may be sampled, and that statistical analysis must be based on "ranking methods"([3]) permitting comparison of the numbers of bacilli in each field with numbers observed in corresponding sites in other films. Fortunately, uniformly replicated microspot films can be made with great rapidity. In the narrow marginal rims of such films the counts tend to be only 2.5 times those encountered across a central plateau.

B. Proper concentrations of protein are required to help retain films on slides.

C. Staining and differentiation must be conducted with reagents that possess "fixing" qualities, in order to minimize the loss of bacilli. Rinsing must be gentle and minimal.

D. The major factors influencing reliability can be demonstrated, tested or controlled by visual inspections, and need not be monitored by tedious compilation of bacterial counts.

[1] Received for publication January 3, 1964.

[2] This work was supported in part by a grant from the World Health Organization and by Grant AI-02998, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service, Bethesda, Maryland.

[3] Leonard Wood Memorial-National Institutes of Health Fellow in Epidemiology, School of Hygiene, Johns Hopkins University.

This paper, therefore, is prepared solely as a technical guide to minimal equipment, maximal convenience, and the factors that must be taken into account to produce reliable results. Since explanations are so tedious in comparison with demonstration, we have tried to illustrate materials, procedures, and the basis of several considerations.

Major features to be described are: the declumping and dispersion of *M. leprae* when necessary, rapid preparation of replicate films on glass slides, safeguards against losses of material during staining and differentiation, and convenient microscopic sampling of films by a method that measures the area covered by each film and eliminates the calibration of microscopic fields.

MATERIALS AND METHODS

Materials include lepromins, tissue homogenates free from rapidly settling tissue particles, and other experimental samples from which uniform aliquots can be drawn. Materials essential to the counting method have been arranged in four groups, three of which are illustrated by figures.

A. Declumping and dispersion of mycobacteria (Fig. 1).—Pointed, 3 ml. glass-stoppered microcentrifuge tubes (Kimball No. 45,152); pure chloroform for declumping; 2 per cent serum in 0.5 per cent phenol water for dispersion and dilution and insurance of uniform retention of films on slides; serologic pipettes for measuring the samples and the chloroform.

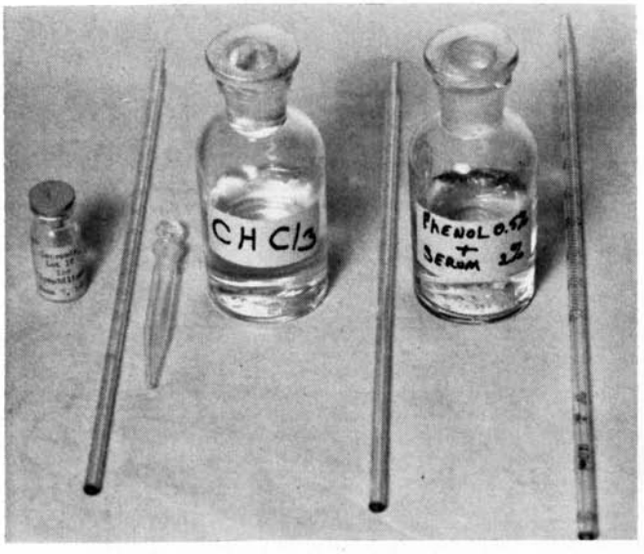

FIG. 1. Materials required for declumping and dispersion of mycobacteria.

B. Preparation of films (Fig. 2).—A spot plate to provide small samples at uniform depths (Arthur H. Thomas, No. 8288, size 2/0); small "sequin" pins for rapid transfer of replicate samples (we use Scoville No. 8-S, selecting for head diameters of 1.53 mm. (0.06") ± 2%); a swab stick, pencil with rubber eraser, or hemostatic clamp to hold the pins by the points; templates for arranging the microspot films in orderly rows on glass slides (prepared by typing "full-stops," "periods," or "o's" on 3" x 5" cards). We now stamp the microspots from each sample as soon as the latter has been placed in the spot plate, thus eliminating the need for rubber stoppers to prevent evaporation.

C. Fixation and staining of films (Fig. 3).—A tightly sealed 70 ml. Coplin jar

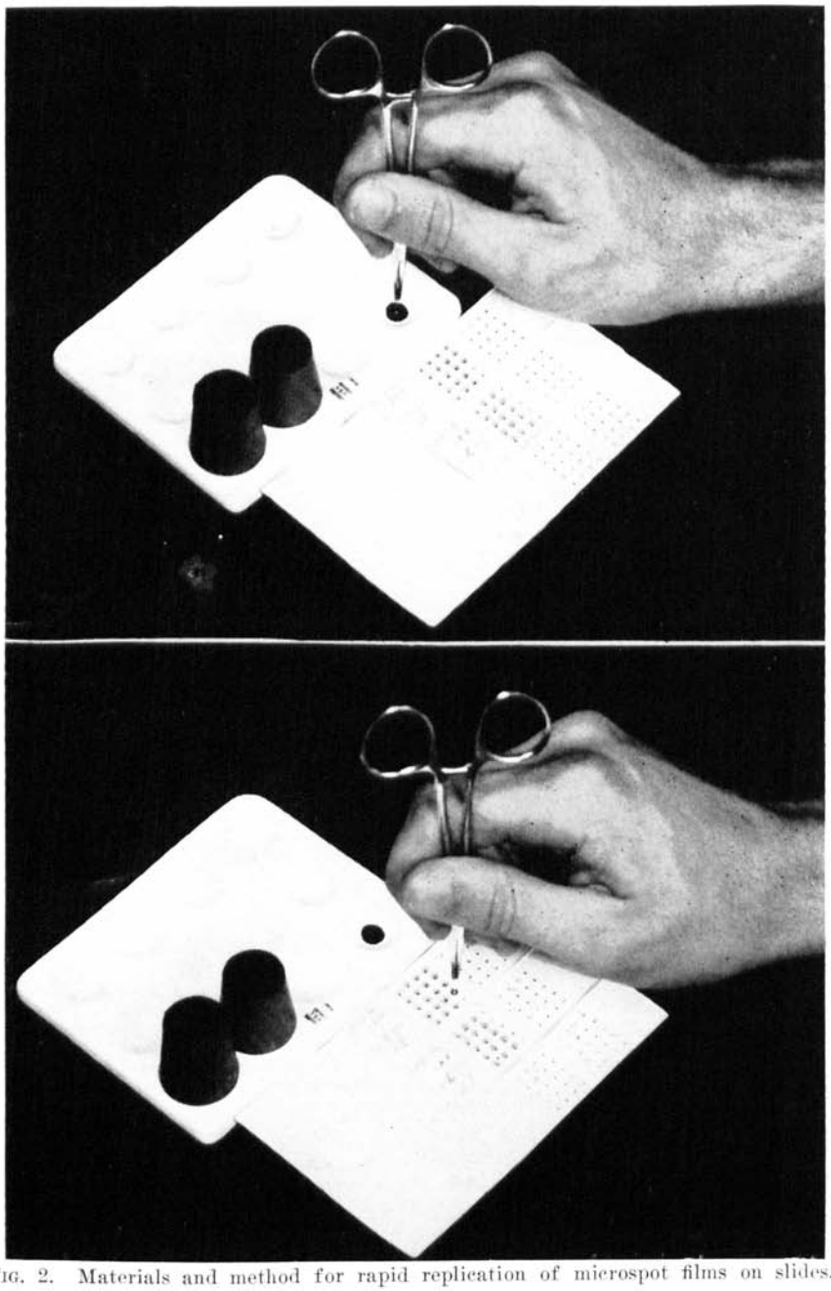

FIG. 2. Materials and method for rapid replication of microspot films on slides.

containing 0.7 ml. of formalin provides 1 per cent of formalin/volume of air. A horizontal glass plate serving as a 98°F. hot plate is laid over a small supply of boiling water in a tin can. Carbolfuchsin in a glass-joint dropping bottle (1 part 3% new fuchsin/wt. in 95% alcohol + 9 parts of 5% phenol in water). Differentiator: a Coplin jar containing 10 ml. of aqueous solution of methylene blue (1%/wt.) and 40 ml. of 5 per cent sulfuric acid/vol. (methylene blue 0.2% in sulfuric acid 4%). For controlled rinsing: a Coplin jar with an appropriate length of nonrusting wire

wedged diagonally across the bottom, to permit slow passage of rinse water beneath the slides; a small funnel to direct the flow of water into the Coplin jar.

D. *Counting.*—A 36-square ocular grid for the eye piece of the microscope (Bausch and Lomb microdiameter disc, Cat. No. 311615). A table of "conversion factors" calculated for the microscope employed.

PROCEDURE

Declumping and dispersion of mycobacteria.—1. Shake the lepromin or other sample thoroughly. Keep it agitated by aspiration while removing 0.3 ml. to a glass-stoppered tube.

2. Add 0.03 ml. chloroform and replace the cap promptly. Immediately shake the contents end to end through an arc of 90° as vigorously as possible, employing 10 strokes for declumping.

3. Immediately add 1.2 ml. of the 2 per cent serum in phenol water and shake twice. The sample has now been diluted five times.

4. To minimize labor, four samples are brought to this point, the stoppers in the tubes are secured with the thumb, and all are shaken vigorously (as described above) for 3 minutes to produce uniform dispersion of the bacilli.

Preparation of films (Fig. 2).—1. Shake and aspirate each dispersed sample and transfer 0.03 ml. to the spot plate.

2. Stir the sample with a pin head as illustrated, and stamp 5 microspots on each of 2 slides. During this operation, the forearm is rested on the table and the wrist is swung in an arc. Superior replications are secured by stamping the pin vertically and boldly on the slide while counting to ensure an even tempo of movement. If for any reason there is a delay in making the microspots on slides, cover the sample with a rubber stopper to prevent evaporation.

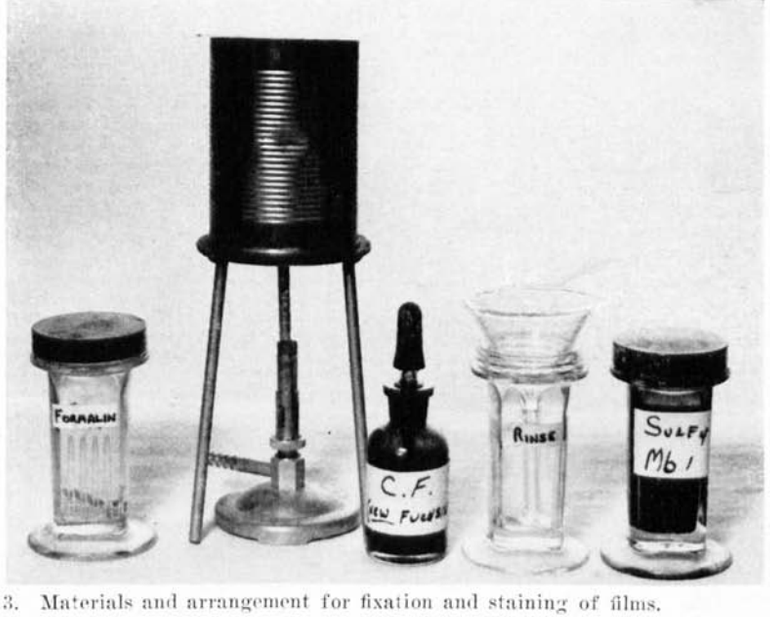

FIG. 3. Materials and arrangement for fixation and staining of films.

As many as 8 vertical rows (samples) are filmed on each slide to ensure uniformity in staining, convenience in labelling, storage, etc. All films are allowed to dry at room temperature.

Fixation and staining of films (Fig. 3).—1. Place the slides in the Coplin jar of formalin vapor for 3 minutes.

2. Lay them on the hot plate for 60 seconds to drive off formalin and to fix by heat.

3. Cover the films with carbolfuchsin for 30 seconds while on the hot plate; rinse *gently* in water; shake off excess water.

4. Immerse the slides in the differentiator for 4 minutes; rinse *gently* in water. Shake off all excess water and allow the slides to air-dry.

The gentle rinsing is carried out by placing the slides in a Coplin jar modified as described and filled with tap water. All films face toward the right. Flowing water is introduced into the left side of the jar through a small funnel. Lest the films be softened, rinsing should be terminated as soon as dyed water has been replaced by clear water.

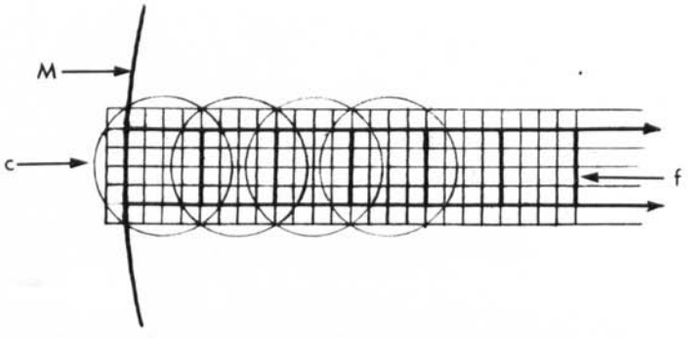

FIG. 4. The use of adjacent square fields to establish the total number of fields and of bacilli per diameter of circular films. **M**—The left margin of a miscrospot film on the slide. **c**—The entire circle of the usual microscopic field, including the 36 small squares into which this area is divided by the ocular. **f**—The actual field of 16 small squares in which the bacilli are counted.

Each movement of the slide is made with a bacillus, particle or other landmark as a reference point, so that exactly one new field is examined for each count. No overlapping or gaps are permitted when moving from field to field.

With a mechanical counter, one key is punched for each field examined. Another key is used to record the number of bacilli. If statistical analysis is intended, the number of bacilli in each field is also recorded in tables prepared for that purpose (3).

Counting of bacilli (Figs. 4 and 5).—1. Restrict enumerations to circular films that have (a) uniform diameters and (b) perfect rims when viewed with the low-power objective. These two requirements are of the utmost importance. Films that are not perfectly matched circles and those with irregular margins indicate a defect in preparation. Films that do not have intact rims, or that have rims with an embroidered or "moth-eaten" appearance, indicate a problem in fixation to slides. They will yield low and discrepant counts.

2. For routine counts, plan to record two items, viz., the number

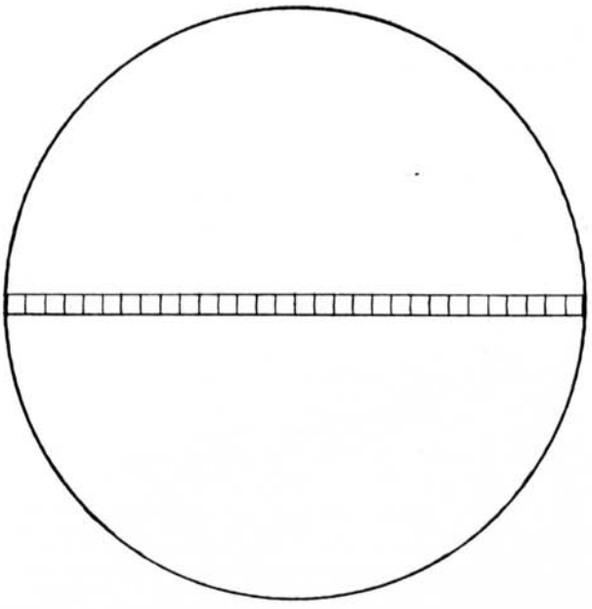

FIG. 5. Geometric relationships between an entire film and the 30 fields that have been examined by method illustrated in Fig 4.

Calculation: The principles can be illustrated step by step on the following basis.

The original sample was diluted 5 times. Each pin head transferred 0.00056 ml. of sample to each microspot. One diameter of the microspot so produced represented 30 fields in linear series, as shown in the figure. In these 30 fields 750 bacilli were found.

By means of the formula πr^2 the total number of fields in the entire microspot is determined. On the basis of 30 fields per diameter, $r = 15$; $r^2 = 225$; and $\pi r^2 = 225 \times 3.1416$. The result is 706 fields in the spot.

The total number of bacilli deposited per pin head, or microspot, is calculated as follows. Since 750 bacilli in a diameter of 30 fields were counted, the average number per field is 25. This number multiplied by the number of fields in the total film (706) gives 17,650 for the number of bacilli in the spot.

The number of bacilli/ml. of the diluted sample is then calculated by multiplying the number of bacilli/pin head spot by the number of pin head deposits constituting 1 ml. Thus $17{,}650 \times \frac{1}{0.00056} = 31{,}500{,}000$, or 31.5×10^6.

The total number of bacilli in the sample is determined by multiplying the latter figure by the dilution factor 5. Thus the total number of bacilli/ml. in the original sample is $5 \times (31.5 \times 10^6)$ or 157×10^6.

As noted previously, once the principle of calculation is understood, the use of conversion factors is recommended.

of square microscopic fields per diameter, and the total bacilli per diameter. The method of starting at the left margin of a film and of counting along a diameter is illustrated in Figures 4 and 5 and explained beneath Figure 4.

3. From your own table of "conversion factors," multiply the appropriate factor by the number of bacilli per diameter. The answer will be expressed as millions of bacilli/ml. The basic principles of calculation are given in an example beneath Figure 5, while the derivation of conversion factors is given under Technical Notes and Discussion.

Criteria for taking into account the differing lengths of bacilli,

the problem of obtaining agreement between counts in replicate films, a statistical method, and determination of the volumes transferred by loops or pin heads, will also be considered under Technical Notes and Discussion.

An alternative approach, adequate for rough comparisons, is simply to stamp carefully replicated films on glass by mean of selected pin heads, to insist on ± 15 per cent agreement between counts in replicate films, and to draw the justified conclusions from direct comparisons between the average numbers of bacilli per diameter.

TECHNICAL NOTES AND DISCUSSION

Papers published previously, of which reprints are available, provide basic data on several phases of the procedures and the counting problem. These emphasize that counting is a tedious and undesirable method for monitoring and controlling the major sources of error. We will call upon this information plus current experience in order to define the basis for essential precautions and recommendations.

A. *Declumping and dispersion.*—These are based on special studies that defined the useful and permissible concentrations of chloroform (1). The pointed tubes are used to minimize the contact between samples and chloroform before shaking can be started.

In studies with lepromin, larger round-bottom tubes and larger samples failed to give equally high counts or as consistent results. In samples that settle rapidly, pipetting larger volumes does not produce the anticipated increases in accuracy. Since Hanks (2) had described the eluting and dispersing effects of 2 per cent serum for recoveries of declumped *M. leprae* from tissue residues, the addition of this diluent immediately after the first 10 vigorous shakes with chloroform was tested. It was shown to be useful. Four possible modifications of procedure were evaluated: shaking by hand and with a Kahn shaker, each with and without the presence of 72 mgm. of Superbrite beads No. 070. The Kahn shaker, producing 60 oscillations per minute, was inadequate, even when operated for 15 minutes. Shaking with the beads for 3 minutes, both by hand and on the Kahn shaker, decreased the bacterial counts. Beads, therefore, were not used further.

With modest concentrations of bacilli in tissues to be studied, declumping should not be necessary. If it is required, homogenates should not be made too dilute since at least 3x dilution is necessary after chloroform to ensure adequate dispersions.

B. *Cleaning of slides.*—Since the microspots of samples must spread uniformly on glass slides, the latter must be scrupulously clean. We immerse or store new slides in a Coplin jar containing 5 per cent glacial acetic acid/vol. in 95 per cent alcohol. Each slide is lifted from the jar, pulled once through a fold of lint-free absorbent tissue (Wipettes, Kimwipes, etc.) and laid on a clean surface.

C. *Preparation of films.*—The transfer of uniformly replicated

samples to glass slides by means of 0.7 mm. loops ([1]) requires considerable practice, both to control volumes and to avoid microspots that are slightly out of round shape because of tremors. Stamping of uniform samples on slides with the pin heads is learned much more easily, gives more reproducible films, and is accomplished much more rapidly. Loops of 28 gauge wire (which is an unusual size) can be obtained only on special order and are expensive. Suitable pins probably can be obtained anywhere. They are used by dressmakers and, in some countries, as paper clips. Atypical pins are discarded.

D. *Monitoring of films without counting.*—Two previous studies ([1, 2]) have demonstrated that the distribution of bacterial concentrations coincides with the distribution of opaque materials (India ink, Congo red, or blood cells) in dried films. The art of making near-perfect replications of microspots should be acquired by critical inspection of films of a sample or of 2 per cent serum containing 1 per cent India ink or 0.2 per cent Congo red.

Inspection of dried films will reveal fairly dense marginal rims surrounding a central plateau. Bacterial counts in the rims tend to be 2.5 times as high as the uniform counts across the central plateau. Should inspection of films or recorded counts show a central peak, the true bacterial numbers may be over-estimated by as much as 20 per cent. This difficulty has been encountered only in homogenates of dense suspensions of tissue cells. It was eliminated by incorporating bile in 0.05 per cent concentration in the dispersing medium ([3]).

The marginal rims and the junctures between these rims and the central plateaus are the regions in which stained films should be examined by low power microscopy, if one suspects problems in film retention.

E. *Calibration of loops and pin heads.*—This has been done by either of two methods, each dependent upon the transfer of test solutions having wetting properties similar to those of lepromin and 2 per cent serum and requiring preliminary practice until almost perfect replication of microspots has been achieved.

1. Titration of alkali *vs* acid: Reagents: 0.05N NaOH, 0.01N HCl, and distilled water, each containing 0.002 per cent phenol red, and hereafter designated as NaOH, HCl and H_2O. The alkali and acid need not be previously "standardized." Add a few drops of an alkaline buffer to 10-20 ml. of the H_2O, adjusting this to the sensitive visual range of phenol red (± pH 7.4). Place ± 1 ml. in a 10 mm. tube to serve as the end-point color standard. By means of serologic pipettes (wiping tips, etc.) titrate the NaOH against the HCl until a conversion factor can be certified. For practice with small volumes, it is convenient to use 0.04 ml. NaOH *vs* ± 0.2 ml. HCl from a serologic pipette. Dilute the acid or alkali so that the HCl required is exactly 5 times the volume of NaOH.

With a 0.7 mm. loop or a pin head transfer 40 microspots of

NaOH to a slide. With the serologic pipette, add HCl to form an essentially neutralized pool of phenol red on the slide. Tip this to a 10 mm. tube containing 0.5 ml. of the H_2O; rinse the slide with 5 drops of H_2O; add more HCl to match the pH color standard in a similar tube. Repeat until the row of small tubes shows essential agreement or permits averaging of results. Calculation: volume of HCl/200 = the volume of NaOH per microspot.

2. Optical density of Congo red solutions: Reagent: 1 per cent Congo red in distilled water. Congo red is chosen for two reasons: its solutions have suitable wetting properties, and negatively charged dyes are not adsorbed by glass. A calibration curve for reading optical densities in the Coleman Jr. spectrophotometer at 500 mμ in 13 mm. tubes was constructed after determining that there is a straight line relationship between optical densities and the per cent concentration of dye over the range suggested. Thereafter two dilutions of the dye served as controls: 0.1 ml. of dye + 49.9 ml. water = 0.002% dye; 0.1 ml. dye stock + 499.9 ml. water = 0.0002% dye. In tubes or cuvettes of greater diameter 0.001% and 0.0001% Congo red may be preferred.

For 0.7 mm. loops: Transfer 10 microspots of 1 per cent Congo red to separate sites on the wall of a clean glass beaker; add exactly 10 ml. of water; pour a sample into the reading tube or cuvette; record the optical density and determine the per cent content of dye from the plotted calibration curve.

For pin heads: From a 0.03 ml. aliquot of 1 per cent Congo red in a spot plate transfer 10 microspots to the end of a clean slide; immerse this end of the slide in 10 ml. of water in a small beaker, and proceed as above.

Calculations: These are based on simple proportions as follows. The volume of 10 microspots: 10 ml. of water = the per cent of dye determined: 1% Congo red. Since the first two elements in this formula cancel, and the last element is 1, the per cent of dye determined by optical density = the volume of solution per microspot in ml. Example: Ten 0.06″ pin heads yield a 0.00056% dye solution. This equals 0.00056 ml. of sample/microspot.

F. *Fixation of films:*—If difficulty is experienced, replace the formalin vapors with formalin in the concentration of 10 per cent in alcohol. Dry the microspots by warming, place the slides in a Coplin jar of the fixative for 10 minutes, and then heat and stain as described. Insoluble materials (India ink or blood cells) can be employed to compare the retention of particles in protein films during exposure to various reagents (1).

G. *Staining and differentiation.*—The problem of losses from the surface of films, the choice of reagents for retention of films, the effect of formalinization on differentiation, determination of end-points for differentiation, and the fact the mycobacteria are not transferred from slide to slide in Coplin jars, have been examined in detail (1).

H. *Factors influencing the accuracy of counts.*—Since counting is employed as a substitute for weight or other estimates of bacterial mass, the variable length of bacilli should be taken into account. We are guided by two considerations. First, typical or average mycobacteria usually contain 4 regularly spaced chromatin bodies or "granules." In the case of *M. leprae*, the length is 2-3μ, a convenient standard for one unit. Bacilli of 4-6μ in length are counted as "two," etc. Secondly, arrangements of branching or conjunctions between bacilli yield "joints," "V's," "W's," "X's," "Y's," etc. Each unit, if not of exceptional length, receives a count of "one." In counting material from treated patients or samples rich in short ovoids or granules, this system would overestimate bacterial mass.

I. *Agreement between counts in replicate films.*—The labor of counting leads to empirical compromises rather than statistical satisfactions. We try to achieve reasonable reliability by counting across only two carefully selected films per sample.

If the counts in a second film agree with the first within $\pm$ 10 per cent, these are averaged, assigning also an average number of fields per diameter of spot. If the two counts disagree by more than 15 per cent, a third film is counted. If two counts now agree within $\pm$ 10 per cent and one disagrees, and reexamination of the latter by low power shows a defective film, that count is disregarded. If all films appear identical, the three counts must be averaged. Careful preparation and inspection of films, therefore, is an important means of saving labor.

In order to obtain this degree of agreement, the numbers of bacilli that must be observed per diameter vary with the material and the mycobacterial species. With uniform distributions of single bacilli in homogenates of *M. lepraemurium*, 200-400 bacilli suffice ([3]). In declumped and dispersed lepromins the number tends to be in the order of 800. In lepromins containing large tissue particles, the required numbers run into the thousands. The example cited below of 750 bacilli per diameter of spot and 158 x 10^6 bacilli/ml. corresponds with the 160 x 10^6 recommended by the 1963 Congress in Rio de Janeiro as a provisional standard for lepromin. In clinical microscopes such lepromins may yield 1,100-1,200 bacilli per diameter of spot. If bacilli are so numerous as to make counting burdensome, work can be saved by recording the numbers of bacilli seen on only two of the four horizontal rows of small squares in the field, and then multiplying by two. When bacilli are in low numbers, a choice must be made between counting additional films or concentrating samples by centrifugation.

The use of convenient statistical methods has been illustrated elsewhere ([3]). The necessary booklet of tables can be obtained from Dr. F. Wilcoxon ([6]). In standardization procedures, we adjust samples to agreement of counts, even though statistical analysis indicates that two counts do not differ significantly.

J. *Calculations and conversion factors.*—The principle employed for calculation of the number of bacilli/ml. of sample is to disregard measurements in millimeters, etc., since the size of each microspot film has been measured in terms of the number of fields per diameter. Irrespective of the microscope used, one-half of this number serves as "r" in the usual formula, πr^2. In this way the calculated area of circle represents the total number of fields covered by the known volume of sample placed in each film. An example of such calculations has been given beneath Figure 5.

Conversion factors: For convenience in routine work we prepare for each microscope a table that requires only that the number of bacilli observed per diameter be multiplied by the appropriate "conversion factor."

1. We do not convert the total bacilli per diameter into average bacilli/field and then multiply by the number of fields per film. The number of fields/film is expressed as *multiples* of the fields/diameter. For example (based on the use of our Leitz Ortholux Microscope):

Fields/film	*Fields/diameter*	= *Multiples*
706	30	23.5
754	31	24.3
804	32	25.1

2. We derive a constant which takes into account the dilution of samples (5x) and the volume of sample per pin head (0.00056 ml.). For example, $5 \div 0.00056 = 8{,}928$.

3. The product of this constant and an appropriate multiple converts the number of bacilli per diameter into bacilli per ml. For example, with 1 bacillus/diameter: 23.5 x 8,928 = 209,808 bacilli/ml. This number when rounded off and expressed as millions = 0.210 x 10^6 bacilli/ml.

An example of the conversion table used for the Leitz Ortholux microscope, therefore is as follows:

Fields/ diameter	Conversion factors	Multiply by bacilli/ diameter	Bacilli/ml.
30	0.210 x 10^6	x 750	= 158 x 10^6
31	0.218 x 10^6	x 750	= 163 x 10^6
32	0.224 x 10^6	x 750	= 168 x 10^6

Since microscopes used in clinical laboratories tend to yield ± 20 fields per diameter, they will yield different conversion factors, which must be calculated by the investigator.

SUMMARY

This paper summarizes the basic considerations, materials and procedures required for rapid and convenient determination of the

concentrations of mycobacteria in clinical and experimental materials. Major features described are the declumping and dispersion of *M. leprae* when necessary, rapid preparation of replicate films on glass slides, safeguards against losses of material during staining and differentiation, and convenient microscopic sampling of films by a procedure measuring the area covered by each film and eliminating the calibration of microscopic fields.

RESUMEN

Este trabajo resume las consideraciones basicas, materiales y procedimientos requeridos para una determinación rapida y conveniente de la concentración de micobacterias en materiales clínicos y experimentales. Los mayores aspectos descriptos son desagrupamiento y dispersion del *M. leprae,* rapida preparación de peliculas replicadas sobre portaobjetos de vidrio, seguridad contra la perdida de material durante la coloración y diferenciación, y conveniente selección de peliculas por un procedimiento que mide las areas cubiertas por cada pelicula y elimina la calibración de campos microscopicos.

RESUMÉ

Cet article résume les considérations de base, l'équipment et la technique pour la détermination rapide et facile des concentrations de mycobactéries dans des échantillons cliniques et expérimentaux. Les points principaux qui sont décrits ont trait à la sèparation et à la dispersion de *M. leprae* lorsque cela s'avere nécessaire, à la préparation rapide de frottis reproductibles sur des lames porte-objects, aux précautions à prendre contre la perte de matérial durant la coloration et la différenciation, et à un facile échantillonnage microscopique du frottis par un procédé qui mesure la surface couverte par chaque frottis et élimine de la sorte la mesure de la superficie des champs microscopiques.

REFERENCES

1. Hanks, J. H. Retention and differentiation of carbolfuchsin-stained mycobacteria in diagnostic films. American Rev. Tuberc. & Pulmon. Dis **74** (1956) 597-607.
2. Hanks, J. H. Quantitative aspects of sampling leprosy skin lesions by the scraped incision method. Internat. J. Leprosy **24** (1956) 424-433.
3. Hanks, J. H. Assay of the fate of mycobacteria in cell and tissue cultures. American Rev. Tuberc. & Pulmon, Dis. **77** (1958) 789-801.
4. Hanks, J. H. Enumeration of *Mycobacterium leprae* for the standardization of lepromin. Internat. J. Leprosy **27** (1959) 134-140.
5. Hanks, J. H. and James, D. F. The enumeration of bacteria by the microscopic method. J. Bact. **39** (1940) 297-305.
6. Wilcoxon, F. Some Rapid Approximate Statistical Procedures. Stamford, Conn., American Cyanamid Co., 1949.