SLIDE CULTURE AS A MEANS OF APPRAISING THE STREPTOMYCIN SENSITIVITY OF TUBERCLE BACILLI IN SPUTUM

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Robert Koch (1882) made the first recorded microscopic study on growing cultures of *M. tuberculosis*, and noted that the organisms formed characteristic serpentine cords at a very early stage in multiplication. Similar appearances have since been described by other observers using a variety of different microculture techniques, in particular by Wright (1924), who suspended tubercle bacilli in blood and incubated the mixture in capillary tubes. This method was used in the first microculture studies on the sensitivity of *M. tuberculosis* to drugs (Fry, 1926; Hesse, Meissner, and Quast, 1928). In 1932 Corper showed that the growth of *M. tuberculosis* in fluid cultures could be detected at a very early stage by the appearance of bacillary fascicles in smears made from the deposit at the bottom of upright culture tubes. Pryce (1941) prepared smears of tuberculous sputum on glass surfaces, incubated these in saponin-lysed blood, and showed that similar microscopic evidence of multiplication could be found in a few days. The growth of contaminants was checked by treatment with dilute acid before incubation. Two important simplifications were introduced by Rosenberg (1943), who used half-slides for preparing smears and replaced lysed blood by Kirchner's (1932) serum-synthetic medium. This technique was used by Muller (1944) in studying the sensitivity of the tubercle bacillus to sulphonamides and other compounds. Inhibition was assessed by comparison with a set of standard smears that had been fixed after varying periods of incubation in a drug-free medium. The first antibiotic examined in this way was penicillin (Iland, 1946). From 1949 onwards a number of papers appeared on the use of slide cultures of sputum to determine the sensitivity of the tubercle bacillus to streptomycin or *p*-aminosalicylic acid (Bernard and Kreis, 1949a, b; Cummings and Drummond, 1949; Gernez-Rieux, Sévin, and Chénet, 1949; Jensen, 1949; Paraf

and Desbordes, 1949; Rubbo, Woodruff, and Morris, 1949; Sievers, 1949; Badoux, 1950; Cummings, Drummond, and Schwartz, 1950; Dissmann and Iglauer, 1950a, b; Freour and Huet, 1950; Pugh, Jones, and Martin, 1950; Rubbo and Morris, 1951; Stangl, 1951; Koenigstein, Cheng, Koenigstein, and Suen, 1951; Uyeda, Yamada, and Okada, 1951). Apart from minor modifications the technique has remained similar to that described by Rosenberg (1943), except that many different culture fluids have been advocated. Opinion is divided mainly between the use of partially defined media of the kind proposed by Kirchner (1932) or Dubos and Noufflard (1950), as against formulae based on lysed or unlysed whole blood. In every case smears are incubated in a series of tubes of fluid medium containing graded concentrations of streptomycin. Incubation is continued until smears in drug-free medium show abundant fascicular micro-colonies when fixed and stained. Most workers have taken as their end-point the absence of bacillary cords, and the majority have made purely subjective comparisons between smears from different drug concentrations. There are references (Bernard and Kreis, 1949a; Sievers, 1949; Rubbo *et al.*, 1949) to smears in which a few organisms show evidence of growth while the majority are unchanged, and this has been interpreted to mean the appearance of "partial resistance" among the bacilli. Rubbo and Morris (1951) report experiments on mixtures of the sensitive strain H37R_v with its resistant variant H37R_v (R), designed to test the ability of slide cultures to distinguish resistant from sensitive elements when both are present. Only Jensen (1949) and Dissmann and Iglauer (1950b) have attempted a numerical, quantitative approach to the comparison of smears. The literature contains little in the way of data on parallel sensitivity determinations by slide culture and alternative techniques

(Gernez-Rieux *et al.*, 1949; Cummings *et al.*, 1950; Rubbo and Morris, 1951). Several passing references are made to the failure of slide culture on sputum containing only small numbers of organisms. Pugh *et al.* (1950) abandoned the use of the method on this account.

As a diagnostic method, culture on slides is certainly inferior to the orthodox method of digestion followed by culture on solid medium, and is probably little more effective in detecting tubercle bacilli than a carefully made direct smear (Oeding, 1951). Cummings (1951) observes that slide cultures are particularly liable to destruction by contaminants, and that they do not distinguish with absolute certainty saprophytic from pathogenic acid-fast bacilli. Wyckoff and Smithburn (1933) have published cinemicrographic studies that show the formation of typical bacillary cords by strains of *Myc. phlei* and mycobacteria from a number of cold-blooded hosts.

The present paper is concerned only with the use of slide cultures to determine the streptomycin sensitivity of tubercle bacilli in sputum.

Materials

The sputa examined were routine samples from the wards of the Westminster Hospital, together with specimens sent in by general practitioners under an arrangement between the hospital and Westminster City Council Department of Public Health. No special method of collection was enjoined. Material from the wards came in waxed cartons. Westminster City specimens were collected in 1 oz. glass vials.

Technical Methods

Bacteriological.—Half-slides were prepared by dividing standard 75 × 25 mm. slides longitudinally. These were boiled in dilute acid, washed with hot, soapy water, rinsed well, dried on a linen cloth, and stored in screw-capped jars.

To prepare a set of cultures from suitable sputum 10 half-slides were marked in pairs with a diamond, according to the concentration of streptomycin in which they would finally be incubated. Two additional slides were marked to serve as fixed controls. The 12 marked slides were flamed thoroughly and set to cool in a sterile 6-in. petri dish, supported on two pieces of 2-mm. glass rod. Each slide in turn was smeared for about two-thirds of its length with sputum from a small pool of selected particles and returned, smear uppermost, to its place in the dish. Fine-pointed forceps were used for handling the sputum. After drying for 20 min. at 36° C. the smears were covered with 12% (v/v) sulphuric acid A.R. by running about 100 ml. of acid directly into the dish. After five minutes the acid was aspirated into a stout 3-litre flask containing 100 ml. of commercial for-

malin. About 100 ml. of sterile saline was then run into the dish. Three changes of saline were made in this way at 5-min. intervals. Corresponding pairs of slides were then placed back to back in tubes of medium containing streptomycin in appropriate concentrations. The two smears designated as fixed controls were removed at this stage, dried, and then heat-fixed. The culture tubes were placed in a 36° C. incubator on a simple rack which held them at an inclination of 20° to the horizontal. In this way a small volume of medium covered the greater part of each slide and exposed a considerable surface to the air inside the tube. Preliminary trials showed that there was no loss in volume during incubation, and no appreciable decline in the streptomycin titre.

Basal Medium.—The basal medium was prepared according to the formula of Dubos and Noufflard (1950):

KH₂PO₄ (anhydrous).—1.0 g.

Na₂HPO₄·12H₂O.—6.3 g.

Asparagine.—2.0 g.

These substances are heated to dissolve in 100 ml. distilled water and then the following are added:

Enzymic Digest of Casein (Difco).—10 ml. of 5% solution (0.5 g.).

Ferric Ammon. Citrate.—1.0 ml. of 5% solution (0.05 g.).

MgSO₄·7H₂O.—1.0 ml. of 1% solution (0.01 g.).

Calcium Chloride.—1.0 ml. of 0.05% solution (0.0005 g.).

ZnSO₄·7H₂O.—1.0 ml. of 0.01% solution (0.0001 g.).

CuSO₄·5H₂O.—1.0 ml. of 0.01% solution (0.0001 g.).

Distilled Water.—850 ml.

The pH is adjusted to 6.6, and the medium is distributed in 4.4-ml. volumes in metal-capped 150×15-mm. rimless hard-glass tubes, and autoclaved for 10 min. at 10 lb./in.²

All component solutions were made in distilled water.

The ferric ammonium citrate solution was prepared freshly on each occasion.

Two supplementary solutions had to be prepared separately and added in appropriate quantities to each tube of basal medium just before use. The first was prepared of 50% glucose in M-100 citric acid sterilized by autoclaving at 5 lb./in.² for 10 min. The second was on oleic acid-albumin complex. Bovine plasma fraction V (Armour) was prepared as a 5% solution in physiological saline and the pH was adjusted to 6.8 to 7.0. Then 0.12 ml. of oleic acid was dissolved by gentle shaking in 10.0 ml. of N/20 sodium hydroxide, and 5.0 ml. of this solution was added to 95 ml. of the 5% bovine plasma albumin. The mixture was sterilized by filtration, distributed in 10-ml. lots, taking care to preserve sterility, and finally heated to 56° for 30 min. to destroy natural lipases.

Culture tubes were prepared for use by adding to each 0.5 ml. of the oleic acid-albumin solution, 0.05 ml. of 50% glucose solution, and appropriate amounts of stock solution of streptomycin to give final concentrations of 1.0 µg. per ml. and 10.0 µg. per ml.

FIG. 1.—Seven-day sputum slide cultures stained by Ziehl-Neelsen-methylene blue ($\times 1,200$): (a) fixed control, (b) incubated control, (c) inhibition by streptomycin ($1 \mu\text{g./ml.}$).

The smears were incubated for six days at 36° . One of the cultures without streptomycin (of which two were originally included in every set) was then removed, 5 ml. of 10% phenol was added and left to act for 30 to 60 minutes. These smears were then stained by the Ziehl-Neelsen method and examined microscopically for evidence of fascicular growth. If numerous fascicles were seen the whole set of cultures was phenolized, stained, and examined. The fixed smears reserved when initiating the test were included at this stage. Fig. 1 (a-c) shows the appearance of typical preparations.

A differential count was now made on each smear, enumerating separately single bacilli and paired or clustered organisms. A few groupings, notably parallel organisms several diameters apart, and organisms lying obliquely across each other, were included in the same category as single bacilli. The object of the differential count was to assess the proportion of organisms in each smear which showed evidence of multiplication ("germinated forms"). The groupings mentioned above do not correspond to any of the early stages in the formation of a micro-colony, but presumably arise by the chance juxtaposition of unrelated organisms occurring when the initial smear is made. These forms and all single bacilli were recorded as "non-germinated forms." On each slide three separate counts of 100 consecutive forms were made, so that there were finally six separate estimates of the proportion of germinated forms for every drug concentration used in the experiment. It is important to note that counts must be done on the fixed controls, since these smears invariably disclosed a proportion of germinated forms, representing organisms that were multiplying at or after the time of expectoration but before the preparation of the culture smears. The difference between proportions of germinated forms in some pairs of cultures was occasionally so large as to be obviously significant. Smaller differences were assessed by calculating the statistic t (Fisher, 1950) and finding from tables the probability (P) that this value would be exceeded by chance. Differences were regarded as significant when P was not greater than 0.01.

TABLE I
RESULTS OF A REPRESENTATIVE SENSITIVITY TEST ON
SPUTUM T 9251 P.18

Controls				Test	
Fixed		Incubated		Streptomycin ($1.0 \mu\text{g./ml.}$)	
Germinated	Non-germinated	Germinated	Non-germinated	Germinated	Non-germinated
(x)		(a)		(b)	
19	81	38	62	17	83
22	78	31	69	11	89
25	75	50	50	23	77
22	78	39	61	14	86
22	78	42	58	18	82
22	78	48	52	13	87
$n-1=6$		$n-1=6$		$n+1=6$	

An example based on the figures given in Table I will serve to illustrate the method of recording and analysis. Comparing the controls (columns x and a):

$$S=5.12; t=6.54; DF=10$$

when $P<0.01$. This difference is regarded as significant, and it is inferred that growth occurred in the incubated control. Now, comparing smears grown in the presence of streptomycin ($1.0 \mu\text{g./ml.}$) with the incubated control (columns a and b)

$$S=6.63; t=6.17; DF=10$$

and $P<0.01$, there is evidence of reduced growth in the presence of this concentration of streptomycin and the strain is regarded as being sensitive to $1.0 \mu\text{g./ml.}$ under the conditions of test.

The selection of a level of significance was necessarily arbitrary, but results justify the value chosen.

Results

The results recorded in Table II were all obtained by the technique described after this had been established through a long series of preliminary trials.

Sixteen specimens from 13 patients yielded viable cultures. Five additional specimens from other patients failed to show evidence of growth

TABLE II
DETERMINATIONS OF SENSITIVITY TO STREPTOMYCIN

Serial No.	Inhibitory Concentration	Streptomycin ($\mu\text{g./ml.}$)
	Slide Culture	Tube Dilution
P/12/28249	>10.0	1.0
P/13/4349	1.0	
P/14/23249	>10.0	
P/15/26549	1.0	
P/16/61250	1.0	0.16
P/17/1251	1.0	
P/18/9251	1.0	0.5
P/19/13251	1.0	2.0
P/20/14251	1.0	1.0
P/21/8349	1.0	
P/22/11451	>10.0	2.0
P/23/4549	1.0	
P/24/6549	1.0	
P/25/191250	1.0	0.3
P/26/41250	1.0	1.8

in the incubated controls and were therefore rejected. In four instances attempts at monthly determinations from the same patient were nullified because organisms became so scanty in the sputum that slide culture was impracticable.

In eight instances the strain tested by slide culture was also isolated from the same sample of sputum by the orthodox methods of digestion and culture. These cultures were then tested for sensitivity to streptomycin in Dubos Tween 80 medium by the tube-dilution technique (Medical Research Council, 1948).

Certain results require additional comment. Specimens P/19 and P/20, P/23 and P/24 were taken from two patients at 48-hour intervals and represent duplicate titrations. Specimens P/14 and P/22 show evidence of resistance and were derived from patients who had received several weeks' treatment with streptomycin alone. Strains P/12 and P/22 show evidence of enhanced sensitivity in the presence of Tween 80. This effect might well have been noted with other strains had the slide cultures been tested with concentrations of streptomycin below 1 $\mu\text{g./ml.}$ The upper limit of 10 $\mu\text{g./ml.}$ was selected because this is generally accepted for clinical purposes as dividing sensitive from significantly resistant strains. Reference to extended titrations is made in the last section of this paper.

It is unfortunate that the series reported does not include any strain showing a high order of resistance in the tube tests, but this was due to the selected type of population from which material had to be drawn.

Discussion

The aim of this investigation was to assess the suitability of slide-culture sensitivity tests for routine use in a clinical laboratory.

A considerable advantage, frequently stressed, is the rapidity of the method in comparison with other techniques. We were able to confirm that unequivocal results can be obtained in less than three days.

Furthermore, the directness of the method avoids that element of selection inherent in all techniques that require isolation of a strain as a prelude to the testing of cultures grown from individual colonies. Every act of isolation on artificial medium implies some degree of adaptation, and this is known in certain instances to relate to drug sensitivity (Lacey, personal communication). It may also be observed that strains from different foci in the same patient may vary widely in their sensitivity (Canetti and Saenz, 1951).

A third important feature of slide culture is that there are no restrictions upon the composition of the medium used. In particular, it is possible to omit surface-active agents intended to promote dispersed growth. Attention has already been drawn to two strains in the series reported here that resisted higher concentrations of streptomycin on slide culture in Dubos basal medium than when tested in tubes of Dubos Tween 80 medium. In 1947 Kirby and Dubos reported a similar phenomenon when testing the penicillin sensitivity of tubercle bacilli. The same effect in relation to streptomycin was described and analysed by Fisher (1948a, b). These original observations have since been confirmed and extended to include other substances (Hauduroy and Rosset, 1948; Suter, Erlenmeyer, Sorkin, and Bloch, 1948; Knight and Tompsett, 1948; Williston and Youmans, 1949; Steenken and Wolinsky, 1949; Yegian and Vanderlinde, 1950; Mitchison, 1950a). Nor does Tween 80 always potentiate the action of drugs against *M. tuberculosis*. Forrest, Hart, and Walker (1947) reported antagonism between Tween 80 and 5 amino-2-butoxypyridine. Other instances of antagonism have been reported (Suter *et al.*, 1948; Eiseman, 1948; Youmans and Youmans, 1948). Schraufstatter (1950) summarized much of the evidence against Tween 80, and concluded that it should never be added to media used for sensitivity tests. Other medium constituents have come under suspicion. Bailey and Cavallito (1950) suggest that long-chain fatty acids may so orient themselves at lipoid surfaces as to leave free anionic groupings that will attract basic molecules such as streptomycin and so enhance the activity of the drug. Mitchison (1950b) claims that streptomycin incorporated into Herrold's (1931) egg medium may partly be bound in an unavailable form. Normal blood con-

stituents may affect the activity of antimicrobial agents (Schraufstatter, 1950), and Bernard and Kreis (1951) report that several strains of *M. tuberculosis* were significantly different in sensitivity when tested by slide culture in blood medium and in serum-synthetic medium. Hirsch (1952) has drawn attention to the effect of medium composition upon the minimal bacteriostatic concentrations of mercuric chloride, o-phenanthroline, "irgafen," dihydrostreptomycin, and penicillin. The use of fluid culture media also avoids inspissation, which is reported to entail partial inactivation of streptomycin (Coletsos, Boisvert, and Oriot, 1950; Drummond, quoted by Youmans, Ibrahim, Sweany, and Sweany, 1950). Nor can it be assumed that streptomycin incorporated into media will retain full activity indefinitely at incubator temperatures. Using solutions of 1,000 $\mu\text{g.}/\text{ml.}$ at a pH between 3 and 7, Regna, Wasselle, and Solomons (1946) found no detectable loss at 28°, but a decline by 33% in seven days at 50°. Pyle (1947) found no loss in the activity of streptomycin in Herrold's medium held for seven weeks at 37°, but Mitchison (1950b) reported losses amounting to 90% in the course of six weeks. Using Dubos Tween medium and assaying supernatant fluid from cultures, Vennesland, Ebert, and Bloch (1947) found losses amounting to almost 90% in four weeks at 37°. The short incubation period needed by slide cultures has therefore technical as well as clinical advantages.

Notwithstanding these considerations, there are two disadvantages which go far toward disqualifying slide cultures of sputum from routine use. Numerous positive sputa failed to give viable cultures. Contamination of glassware with toxic trace substances (Drea, 1942), culture aeration, and age of material were carefully investigated without any clue being uncovered. Berry and Lowry (1949) refer to nine failures in 88 cultures from positive sputa, and Cummings and Drummond (1949) speak of erratic results. It is interesting to recall in this connexion recent reports on failure to demonstrate viable tubercle bacilli from areas of resected lung lesions which gave positive direct smears (Beck and Yegian, 1952; Medlar, Bernstein, and Steward, 1952). There is also evidence from other sources that 20 to 25% of sputa yielding scantily positive direct smears may yet fail to give positive results on culture or animal inoculation (Public Health Laboratory Service, 1952; Cruickshank, 1952). The use of the method is also limited to sputa containing appreciable numbers of bacilli. Attempts at serial examination during treatment have failed because organ-

isms became too scanty. This was noted as a practical disadvantage by Pugh *et al.* (1950), and noted, though not rated a drawback, by Dissmann and Iglauer (1950a).

From a more academic point of view, slide culture affords a potentially interesting approach to the general problem of bacterial sensitivity to drugs. Hitherto attention has been mainly devoted to attempts at determining, for each drug and organism, a single threshold concentration that would draw a sharp distinction between sensitive and resistant strains. Evidence from many sources (Alexander, 1949) now supports the view that sensitivity to drugs among bacteria is a continuously variable quality that is best represented in terms of frequency distribution diagrams. Any population comprising bacteria of only a single strain may be expected to include a very large number of individuals whose sensitivities cluster closely about a modal value, together with progressively fewer organisms having sensitivities much above or much below the mode. For the tubercle bacillus, many reports testify to the existence of very infrequent individuals of very high resistance among populations drawn from strains thought to be fully sensitive to streptomycin (Pyle, 1947; Vennesland *et al.*, 1947; Yegian and Vanderlinde, 1948, 1950; Karlson and Needham, 1948; Fisher, 1948b; Yegian, Budd, and Vanderlinde, 1949; Mitchison, 1950b). This view brings resistance to drugs into line with other biological qualities of bacteria (Chick, 1930; Gardner, 1931; Withell, 1942; Jordan and Jacobs, 1944), and forms a link with the behaviour of other living orders (Finney, 1947). Current macroscopic methods of testing the drug sensitivity of bacteria stress the modal value, but give little or no information concerning the scatter of values on either side. Jensen (1949) refers specifically to the fact that slide culture gives an indication of the relative proportions of resistant and sensitive organisms, which is lacking in the data from orthodox tube-dilution tests. There is needed a means of appraising whole populations, rather than methods bringing added refinement to the determination of an arbitrary, and largely illusory, end-point.

Data for constructing drug-sensitivity distribution curves can be obtained from multiple surface-viable counts, but these present particular technical difficulties when dealing with the tubercle bacillus. Slide culture offers an elegant alternative. From simple mathematical considerations an expression can be derived which relates the proportion (p) of viable organisms killed by exposure to a given con-

centration of drug to the proportions of germinated forms found in slide cultures. This proportion killed (p) may either be plotted against drug concentration, or converted to probits and plotted against the logarithm of drug concentration. This latter presentation has the advantage of being approximately linear. The results of culture of sputum in smears are not well suited to analysis in this way owing to the high proportion of germinated forms in fixed controls. The figures in Table III are taken from a representative slide-

TABLE III
RESULTS OF A REPRESENTATIVE SLIDE-CULTURE EXPERIMENT

Streptomycin Concentration ($\mu\text{g./ml.}$)	Log Concentration	Percentage Germinated Forms (K)	Percentage Killed ($K-c/c'-c$)	Probit
0.0625 ..	2.8	81.4	5.0	3.36
0.125 ..	1.1	80.0	7.9	3.59
0.25 ..	1.4	49.8	67.8	5.46
0.50 ..	1.7	45.2	76.9	5.74
1.0 ..	0.0	43.5	98.0	7.05
2.0 ..	0.3	35.5	99.6	7.65
Incubated control (c') ..	—	84.0	—	—
Fixed control (c) ..	—	33.6	—	—

culture experiment using a specially prepared suspension of tubercle bacilli instead of positive sputum. The accompanying graphs (Figs. 2 and 3) show the approximate form of the distribution

FIG. 2.—Relationship between log concentration of streptomycin and proportion of organisms killed expressed as probits.

FIG. 3.—Cumulative frequency diagram relating proportions of organisms killed to log concentration of streptomycin.

of sensitivity. A regression line has been fitted to the probit values by the method of least squares.

It will clearly be of interest to attempt the correlation of this kind of experiment with clinical studies. It might well be the case that information on sensitivity scatter would be more useful in predicting the clinical outcome of treatment than the modal value, and parameters for probit-log dose regression lines may perhaps prove to be more useful indices of sensitivity for clinical purposes than the current threshold type of value. Alternatively, such regression lines can be used to give precision to threshold values by enabling an LD50 or LD90 to be estimated. If the LD50 proves to be useful in practice, it can rapidly be computed by the method of "moving averages" (Thompson, 1947) without recourse to the labour of calculating probits and fitting regression lines.

Summary

A technique is described by which the sensitivity of tubercle bacilli in sputum to streptomycin can be appraised directly.

Eight strains of *M. tuberculosis* were tested in parallel by slide culture and a tube-dilution technique.

The method of slide culture of sputum possesses considerable advantages, but is unsuited to routine use because of the irregular viability of the smears.

A quantitative approach to sensitivity is discussed, and examples are given of the application of slide culture to the appraisal of bacterial sensitivity to drugs as a population characteristic with a typical frequency-distribution form.

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REFERENCES

- Alexander, H. E. (1949). *Evaluation of Chemotherapeutic Agents*, ed. MacLeod, C. M. (N.Y. Acad. Med. Sect. Microbiol. Symposium No. 2). Columbia University Press, New York.
- Badoux, V. (1950). *Bull. Soc. Vaud. Sci. Nat.*, **64**, 479.
- Bailey, J. H., and Cavallito, C. J. (1950). *J. Bact.*, **60**, 269.
- Beck, F., and Yegian, D. (1952). *Amer. Rev. Tuberc.*, **66**, 44.
- Bernard, E., and Kreis, B. (1949a). *Ann. Inst. Pasteur*, **77**, 653.
- (1949b). *Rev. Tuberc.*, **13**, 124.
- (1951). *Ann. Inst. Pasteur*, **80**, 315.
- Berry, J. W., and Lowry, H. (1949). *Amer. Rev. Tuberc.*, **60**, 51.
- Canetti, G., and Saenz, A. (1951). *Ann. Inst. Pasteur*, **80**, 238.
- Chick, H. (1930). *A System of Bacteriology in Relation to Medicine*, vol. 1, p. 185. Medical Research Council. H.M.S.O., London.
- Coletos, P. J., Boisvert, H., and Oriot, E. (1950). *Ann. Inst. Pasteur*, **79**, 288.
- Corper, H. J. (1932). *J. Amer. med. Ass.*, **99**, 1315.
- Cruikshank, D. B. (1952). In *Modern Practice in Tuberculosis*, vol. 1, p. 55, ed. Sellors, T. H., and Livingstone, J. L. Butterworth, London.
- Cummings, M. M. (1951). *Amer. J. clin. Path.*, **21**, 684.
- and Drummond, M. (1949). *Amer. Rev. Tuberc.*, **59**, 599.
- and Schwartz, H. B. (1950). *Dis. Chest*, **17**, 202.
- Dissmann, E., and Iglaue, E. (1950a). *Wien. klin. Wschr.*, **62**, 847.
- (1950b). *Tuberkulosearzt*, **4**, 629.
- Drea, W. F. (1942). *J. Bact.*, **44**, 149.
- Dubos, R., and Noufflard, H. (1950). *Ann. Inst. Pasteur*, **78**, 208.
- Eiseman, B. (1948). *J. exp. Med.*, **88**, 189.
- Finney, D. J. (1947). *Probit Analysis*. University Press, Cambridge.
- Fisher, M. W. (1948a). *Amer. Rev. Tuberc.*, **57**, 53.
- (1948b). *Tuberculosis*, **10**, 18.
- Fisher, R. A. (1950). *Statistical Methods for Research Workers*, 11th ed., p. 122. Oliver and Boyd, Edinburgh.
- Forrest, H. S., Hart, P. D'A., and Walker, J. (1947). *Nature, Lond.*, **160**, 94.
- Freour, P., and Huet, M. (1950). *J. Méd. Bordeaux*, **127**, 487.
- Fry, R. M. (1926). *Brit. J. exp. Path.*, **7**, 174.
- Gardner, A. D. (1931). *Microbes and Ultramicrobes*, p. 15. Methuen, London.
- Gernez-Rieux, C., Sévin, A., and Chénét, C. (1949). *Ann. Inst. Pasteur, Lille*, **2**, 57.
- Hauduroy, P., and Rosset, W. (1948). *C.R. Soc. Biol., Paris*, **142**, 484.
- Herrold, R. D. (1931). *J. infect. Dis.*, **48**, 236.
- Hesse, E., Meissner, G., and Quast, G. (1928). *Arch. exp. Path. Pharmac.*, **135**, 82.
- Hirsch, J. (1952). *Zbl. Bakt., I Abt.*, **158**, 219.
- Iland, C. N. (1946). *J. Path. Bact.*, **58**, 495.
- Jensen, K. A. (1949). *Acta tuberc. scand. Suppl.* 21, p. 42.
- Jordan, R. C., and Jacobs, S. E. (1944). *J. Hyg., Camb.*, **43**, 275.
- Karlson, A. G., and Needham, G. M. (1948). *Proc. Mayo Clin.*, **23**, 401.
- Kirby, W. M. M., and Dubos, R. J. (1947). *Proc. Soc. exp. Biol., N.Y.*, **66**, 120.
- Kirchner, O. (1932). *Zbl. Bakt., I Abt.*, **124**, 403.
- Knight, V., and Tompsett, R. (1948). *J. clin. Invest.*, **27**, 544.
- Koch, R. (1882). *Berl. klin. Wschr.*, **19**, 221.
- Koenigstein, R. P., Cheng, P. S., Koenigstein, L., and Suen, I. D. (1951). *Wien. klin. Wschr.*, **63**, 723.
- Medical Research Council (1948). *Lancet*, **2**, 862.
- Medlar, E. M., Bernstein, S., and Steward, D. M. (1952). *Amer. Rev. Tuberc.*, **66**, 36.
- Mitchison, D. A. (1950a). *Thorax*, **5**, 162.
- (1950b). *Ibid.*, **5**, 144.
- Muller, H. (1944). *J. Path. Bact.*, **56**, 429.
- Oeding, P. (1951). *Acta tuberc. scand.*, **25**, 208.
- Paraf, J., and Desbordes, J. (1949). *Bull. Soc. méd. Hôp. Paris*, **65**, 1355.
- Pryce, D. M. (1941). *J. Path. Bact.*, **53**, 327.
- Public Health Laboratory Service, Working Party (1952). *Monthly Bull. Minist. Hlth, Lond.*, **11**, 187.
- Pugh, D. L., Jones, E. R., and Martin, W. J. (1950). *Lancet*, **2**, 92.
- Pyle, M. M. (1947). *Proc. Mayo Clin.*, **22**, 465.
- Regna, P. P., Wasselle, L. A., and Solomons, I. A. (1946). *J. biol. Chem.*, **165**, 631.
- Rosenberg, K. S. (1943). *Lancet*, **1**, 615.
- Rubbo, S. D., and Morris, D. M. (1951). *Journal of Clinical Pathology*, **4**, 173.
- Woodruff, P. S., and Morris, D. M. (1949). *Nature, Lond.*, **164**, 313.
- Schraufstatter, E. (1950). *Z. Hyg. InfektKr.*, **131**, 318.
- Sievers, O. (1949). *Lancet*, **1**, 798.
- Stangl, E. (1951). *Wien. klin. Wschr.*, **63**, 691.
- Steenken, W., and Woinsky, E. (1949). *J. Bact.*, **57**, 453.
- Suter, E., Erlenmeyer, H., Sorkin, E., and Bloch, H. (1948). *Schweiz. Z. Path. Bakt.*, **11**, 193.
- Thompson, W. R. (1947). *Bact. Rev.*, **11**, 115.
- Uyeda, S., Yamada, O., and Okada, H. (1951). *Acta tuberc. japonica*, **1**, 1.
- Vennesland, K., Ebert, R. H., and Bloch, R. G. (1947). *Science*, **106**, 476.
- Williston, E. H., and Youmans, G. P. (1949). *Amer. Rev. Tuberc.*, **59**, 336.
- Withell, E. R. (1942). *J. Hyg., Camb.*, **42**, 124, 339.
- Wright, A. E. (1924). *Lancet*, **1**, 218.
- Wyckoff, R. W. G., and Smithburn, K. C. (1933). *J. infect. Dis.*, **53**, 201.
- Yegian, D., Budd, V., and Vanderlinde, R. J. (1949). *J. Bact.*, **58**, 257.
- and Vanderlinde, R. J. (1948). *Ibid.*, **56**, 177.
- (1950). *Amer. Rev. Tuberc.*, **61**, 483.
- Youmans, A. S., and Youmans, G. P. (1948). *J. Bact.*, **56**, 245.
- Youmans, G. P., Ibrahim, A., Sweany, J., and Sweany, H. C. (1950). *Amer. Rev. Tuberc.*, **61**, 569.