APPLIED MICROBIOLOGY, Sept. 1972, p. 311-317

Vol. 24, No. 3
Printed in U.S.A.

Long-Term Preservation and Storage of Mycobacteria

THOMAS H. KIM AND GEORGE P. KUBICA
Trudeau Institute, Inc., Saranac Lake, New York 12983

Received for publication 13 April 1972

Under contract with the National Institute of Allergy and Infectious Diseases, the Trudeau Mycobacterial Culture Collection has been greatly expanded to provide for the scientific community a collection of representative strains of mycobacteria of biomedical importance. Problems concerned with the preparation, bottling, and distribution of such organisms have been dealt with and are detailed in this paper. Examination of collected data revealed that the temperature of storage and not the suspending menstruum was more important for prolonged survival of mycobacteria stored at subzero temperatures. For optimum results, mycobacteria may be suspended either in Dubos Tween-albumin broth or in Middlebrook 7H-9 liquid medium supplemented with ADC enrichment (commercial sources used) and stored at −70 C. Either of these suspending fluids supplies a growth-supporting medium for cultures which must be shipped long distances, often without refrigeration. To avoid sublimation of suspending medium during prolonged storage at −70 C (a problem inherent in many screw-capped containers), we have chosen to use vaccine-stoppered serum bottles sealed with aluminum crimp caps. The methods described have provided suspensions with (i) excellent viability over prolonged periods of storage, (ii) stable metabolic activities, and (iii) highly reproducible inocula for animal experiments.

In 1966 the National Institute of Allergy and Infectious Diseases and the American Thoracic Society held a Conference on the Laboratory Evaluation of Immunization Against Tuberculosis. One of the recommendations of this conference was that a mycobacterial culture collection be established with the following objectives: (i) to collect representative strains of biomedically important mycobacteria; (ii) to characterize these strains with respect to taxonomic classification, infectivity, and virulence; (iii) to determine optimal methods for preservation of the cultures; and (iv) to distribute these well-characterized strains to qualified investigators for use in health-related research.

Such a collection has been established at this institute, and a catalog describing the strains will soon be available (requests for Trudeau Mycobacterial Culture Collection Catalog: Program Officer, Tuberculosis Panel, U.S.-Japan Program, Geographic Medicine Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20014). To provide the above suggested services to the scientific community, it was obvious that some method of preservation and storage would have to be developed which would (i) eliminate technical difficulties associated with constant transfer of cultures, (ii) minimize the risk of attenuation in virulence often associated with repeated transfers on culture media (10), (iii) maintain culture viability and metabolic activity for prolonged periods of storage, and (iv) retain maximal viability of cultures during shipment through the mails. Of incidental importance to the culture collection, but of vital importance to proposed animal studies was the need to retain essentially 100% viability of vaccinating or challenge suspensions, or both, of mycobacteria. This was necessary to insure reproducible and precise levels of infection from one animal experiment to the next. Photometric and gravimetric procedures were totally unsuitable for standardization of viable counts since they measured only the numbers of particles in suspension without regard for their viability.

Ideally, lyophilization would be the method of choice for culture storage, since *Mycobacterium bovis* BCG has been shown to retain high levels of viability and immunogenicity

when stored in the freeze-dried state (3, 12). Certainly, this method has been successfully used in recent studies (14; Kubica et al. J. Gen. Microbiol., *in press*). However, one recent report (2) attributed the loss in virulence of *M. intracellulare* to the selection by culture, or suppression by lyophilization, of the more virulent smooth, transparent colony form. Since several of our staff had successfully employed the method of low-temperature freezing described by Grover et al. (4), this seemed like the logical starting point. This report describes our experience with low-temperature storage for preservation of viability and virulence of mycobacteria.

MATERIALS AND METHODS

Initial studies were conducted to determine the effect on microbial viability of two factors: the temperature of storage and the suspending fluid. *M. tuberculosis* strain H37Rv and *M. bovis* BCG were grown as thin rapidly spreading pellicles; the former organism was grown on the surface of Proskauer and Beck (PB) medium (7, 15), whereas the BCG strain was grown on Sauton's medium (see Trudeau Mycobacterial Culture Collection Catalog). Heavy suspensions of these two organisms were prepared to contain approximately 10^7 to 10^8 viable cells per ml by homogenizing the pellicle in Teflon-glass homogenizers. The cell suspension was divided into nine samples, and each was washed three times in distilled water. The washed cell suspensions were then resuspended in one of the nine diluents listed below. At this point each H37Rv suspension was subjected to a 3-sec burst of sonic treatment at maximum output on a Bronwill Biosonic II to provide a suspension composed essentially of single cells. Because an earlier experiment (*unpublished data*) had shown even brief periods of sonic treatment to be harmful to BCG, the sonic oscillator was not used on suspensions of this organism. Suspensions of both species were finally diluted 1:10 in one of the nine diluents; all (except skim milk suspensions) were photometrically adjusted to provide approximately 10^6 to 10^7 cells per ml, and the suspensions were aseptically dispensed in 1-ml amounts into 2-ml capacity serum bottles. These bottles were closed with flange stoppers and one-piece aluminum crimp seals. The bottled suspensions were divided into two batches, one to be stored at −20 C, the other at −70 C. At periodic intervals, randomly selected bottles were removed from each deep freezer, rapidly thawed in a 37 C bath, and plated in triplicate on OADC-enriched Middlebrook 7H-10 agar (commercial sources satisfactory) for viable count.

The nine diluents examined were (no. 1) sterile skim milk (5); (no. 2) 1% gelatin buffered at *p*H 6.8 (1); (no. 3) 1:5 dilution of Sauton's medium; (no. 4) 0.25% Triton WR 1339 in Sauton's medium (D. Wright, *personal communication*); (no. 5) a solution containing 8.3% dextran, 7.5% glucose, and 0.025% Triton WR 1339 (13); (no. 6) 15% aqueous solution of lactose at *p*H 5.0 (A. Frappier, *personal communication*); (no. 7) 5% sodium glutamate (T. Sawada, *personal communication*); (no. 8) Tween-albumin medium (4); (no. 9) distilled water (6).

Virulence testing. Prior experience had taught that progressive reduction (in 10-fold increments) of the number of viable units of tubercle bacilli injected into guinea pigs resulted in a decrease in the virulence index for this animal as determined by the method of Steenken and Gardner (11). This method involves the subcutaneous injection of 10^5 to 10^6 viable tubercle bacilli into five guinea pigs. After 12 weeks the animals are sacrificed and virulence is subjectively assessed as the sum of the graded numerical involvement of gross disease observed in lungs, spleens, and lymph nodes. Each of these organs is permitted a range from 0 to 4, so that widespread tuberculous involvement of all organs would result in a virulence index of 12. Organisms could then be placed into one of three categories: high virulence (index 9 to 12); moderate virulence (index 5 to 8); or low virulence (index 1 to 4). One of our strains of *M. tuberculosis* exhibited a virulence index of 9 when 10^6 organisms were injected into guinea pigs. Since this value is on the border between high and moderate virulence, we reasoned that any marked loss in viability of the organism as a result of prolonged storage might be reflected in a depression of the virulence index. Accordingly, a suspension of this organism, containing 10^6 viable units per 0.2 ml (the amount injected into guinea pigs), was prepared in diluent 2 (1% buffered gelatin) and dispensed into replicate vials, half of which were stored at −20 C and half at −70 C. At periodic intervals vials were removed from the two freezers, thawed, and injected into groups of five guinea pigs. Concomitantly, viable counts were performed by plating suitable 10-fold dilutions of the thawed suspensions.

RESULTS

In Tables 1 and 2 are recorded the observed viable counts for H37Rv and BCG stored in nine different diluents and at two temperatures (−20 C and −70 C) for a period of 3 years. Selected data from Tables 1 and 2 have been used to illustrate graphically the survival of H37Rv and BCG in four of the nine diluents under test (see Fig. 1).

It is obvious that storage at −20 C in any vehicle is detrimental to the long-term survival of these two species of mycobacteria; in some instances less than 0.0002% of the starting suspension is still viable, and 13 of the 18 samples examined after 3 years showed only 1% or fewer viable bacilli remaining (see Tables 1 and 2). The number of organisms surviving the two storage temperatures also is reflected in the "apparent virulence" of *M. tuberculosis*, as compared in Fig. 2. The viability of the inoculum stored at −70 C (Fig. 2A) maintained at essentially 10^6 per 0.2 ml for a full year, and the virulence index

varied between 8.6 and 9.2, except for the single low index of 7.6 at month 4. The fact that both prior and subsequent indexes were around 9.0 has caused us to attribute the virulence index of 7.6 to one of those occasional inexplicable variations expected and regretfully accepted in biological sciences. In contrast the bacillary population stored at −20 C (Fig. 2B) showed a gradual reduction in numbers from 10^6 to 10^3, which was reflected as a suppression in "apparent" virulence index from 9.1 to 4.6. That this organism had not lost virulence could be demonstrated by subculturing the bacilli to attain a viable population of 10^6 for reinoculation into guinea pigs, and restoration of the 9.0 level of virulence. The fact that there was no loss in virulence as a result of storage at −20 C supports an earlier

TABLE 1. *Observed viable counts of Mycobacterium tuberculosis H37Rv stored for 3 years in nine different suspending media and at two different temperatures*[a]

Temp in suspending medium	Months of storage	1	2	3	4	5	6	7	8	9
−70 C	0	1.0	0.9	1.1	0.8	0.4	0.6	0.7	0.9	0.8
	1	1.5	1.2	1.2	1.1	0.8	0.8	0.5	0.8	0.6
	2	1.0	1.6	1.3	1.5	1.1	0.4	0.6	0.9	0.7
	4	2.0	1.7	1.5	1.3	1.6	1.5	1.0	1.0	1.1
	8	1.2	0.8	0.9	1.5	0.7	0.5	0.5	0.7	0.8
	12	1.0	0.7	1.0	1.1	0.9	0.7	0.6	0.8	0.5
	20	1.8	1.0	1.7	1.6	1.5	0.9	0.6	0.7	0.7
	36	1.4	0.9	1.2	1.0	0.9	0.7	0.5	1.1	0.2
−20 C	0	1.0	0.9	1.0	0.8	0.4	0.6	0.7	0.9	0.8
	1	1.0	1.1	0.8	0.6	0.7	0.8	0.5	0.8	0.4
	2	1.1	0.3	0.6	0.4	0.7	0.4	0.6	0.9	0.07
	4	1.0	0.3	0.9	0.6	0.5	0.4	0.7	0.5	0.003
	8	0.2	0.01	0.3	0.3	CT	0.02	0.2	0.1	0.0003
	12	0.05	0.0004	0.09	0.2	0.08	0.0005	0.07	0.05	6×10^{-5}
	20	0.04	0.0002	0.004	0.1	0.05	0.0002	0.08	0.07	3×10^{-5}
	36	0.05	0.0004	0.004	0.07	0.02	1×10^{-6}	0.03	0.02	5×10^{-5}

[a] All figures indicate observed counts $\times 10^{-7}$. Check text for identity of suspending media numbered 1 to 9. CT = contaminated.

TABLE 2. *Observed viable counts of Mycobacterium bovis BCG stored for 3 years in nine different suspending media and at two different temperatures*[a]

Temp in suspending medium	Months of storage	1	2	3	4	5	6	7	8	9
−70 C	0	7.5	6.1	3.5	7.4	4.7	5.0	7.0	8.6	7.8
	1	10.0	5.6	4.2	4.2	1.6	2.9	2.1	4.5	2.0
	2	5.4	4.4	5.0	5.0	2.6	1.7	2.2	4.2	1.9
	4	3.5	4.4	2.4	3.7	1.2	1.9	1.7	2.0	1.8
	8	6.0	2.2	6.1	6.7	2.0	2.0	2.2	5.4	0.2
	12	6.6	5.0	6.8	7.0	1.9	3.8	3.2	6.0	0.7
	20	7.1	3.6	5.7	6.3	4.1	3.8	2.9	4.6	0.09
	36	2.0	1.4	4.1	5.1	2.0	1.8	1.7	2.2	0.2
−20 C	0	7.5	6.1	3.5	7.4	4.7	5.0	7.0	8.6	7.8
	1	5.9	2.1	3.5	4.7	3.7	1.5	2.1	4.0	1.6
	2	0.9	0.03	1.9	2.1	0.9	0.3	0.6	1.0	0.3
	4	0.7	0.6	0.9	0.9	0.7	0.1	0.4	0.5	0.03
	8	0.05	0.005	0.4	0.8	0.1	0.03	0.04	0.06	0.02
	12	0.05	0.001	0.4	1.6	0.08	0.08	0.05	0.01	0.002
	20	0.007	0.005	0.08	0.2	0.06	0.001	0.04	0.003	0.0002
	36	0.004	0.0002	0.02	0.08	0.02	4×10^{-5}	0.01	0.002	1×10^{-5}

[a] All figures indicate observed counts $\times 10^{-6}$. See text for identity of suspending media numbered 1 to 9.

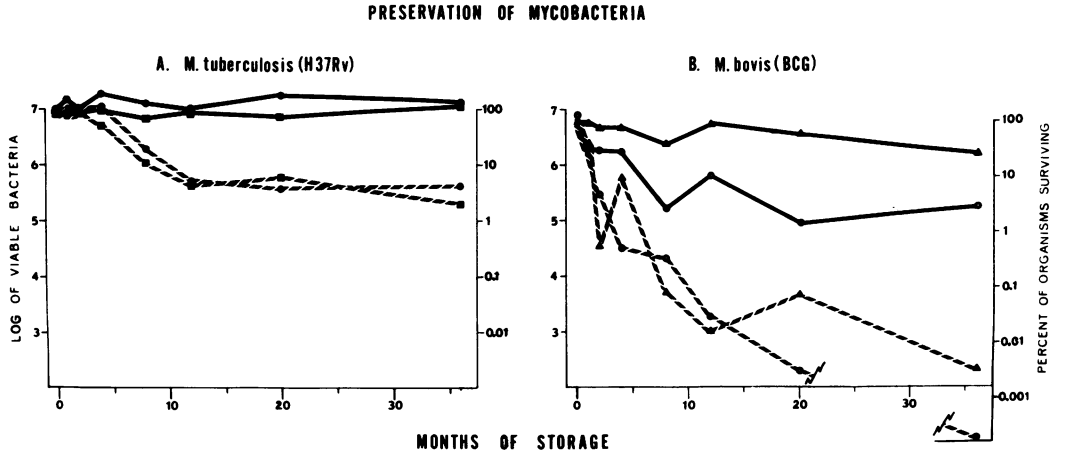

FIG. 1. *Long-term preservation of (A) M. tuberculosis and (B) M. bovis (BCG) stored at −70 C and −20 C in different diluents. In both diagrams survival at −70 C is indicated in solid lines, whereas −20 C is shown in broken lines. Diluents chosen for demonstration are: skim milk (●), Tween-albumin medium (■), phosphate-buffered gelatin (pH 6.8) (▲), and distilled water (○). See text for details.*

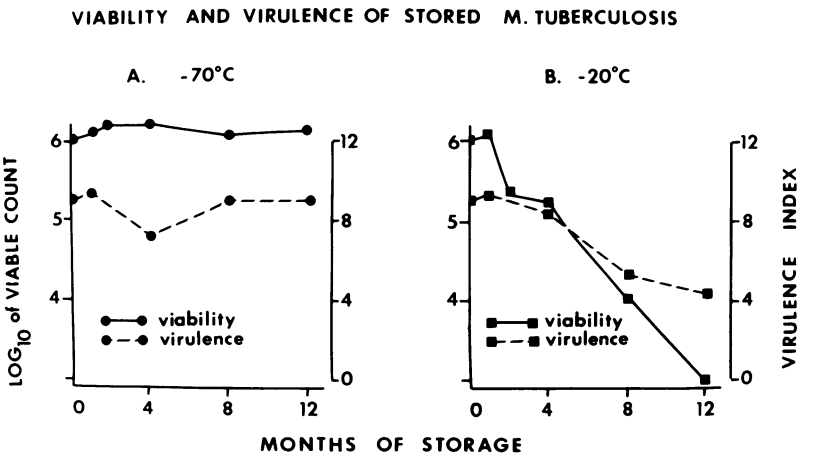

FIG. 2. *Effect on viability and "apparent" virulence of M. tuberculosis stored at −70 C (A) and −20 C (B).*

report of Mitchison et al. (9).

The BCG strain (Fig. 1B), which was not sonically treated, tended to clump more in some diluents than in others, resulting in "see-saw" viability curves. This emphasized to us the need for suspension and storage of inoculum in a vehicle which would maintain the cells at least as well dispersed as was the starting suspension. It also has encouraged further studies on methods for preparation of a more dispersed inoculum of the possibly sonic-treatment-sensitive *M. bovis* BCG.

Examination of the data for strain H37Rv, which had more uniform plate counts over the prolonged storage period, would support the observation of Grover et al. (4), that the storage temperature, and not the suspending medium, is more important for prolonged survival of *M. tuberculosis* and *M. bovis* BCG. With the possible exception of distilled water (diluent 9) all other suspending fluids appeared to retain viability of mycobacteria for several years at −70 C. A pilot study (*unpublished data*) revealed that various species of mycobacteria, suspended in Middlebrook 7H-9 broth (commercial sources), could be shipped distances in excess of 5,000 miles, over periods of time ranging from 4 to 10 days, without appreciable loss of viability. It appeared then that Middlebrook 7H-9 broth would satisfy our need for a suspending-storage-shipping medium.

Our next series of problems revolved around the need to (i) provide a safe, mass-production

bottling procedure for the staff engaged in the maintenance and distribution of this culture collection, (ii) utilize a bottle which would minimize loss of diluent by sublimation during prolonged storage at freezer temperatures, (iii) employ a bottle which would survive the harsh treatment encountered in shipment through the mails, (iv) minimize the chances of aerosol infection when attempting to recover the bacterial suspension from the bottles.

Problems ii to iv were resolved by utilizing bottles and closures available from Wheaton Scientific, Millville, N.J. We chose thick-walled borosilicate glass serum bottles (Vitro "400") of 1.8 to 2.0-ml capacity with matching rubber flange stoppers and one-piece aluminum crimp seals (see Fig. 3). We found that we could stopper and crimp these vials, remove the "punch out" center of the aluminum crimp, and sterilize by autoclaving at 121 C for 30 min.

The problem of mass-production bottling was effectively handled by construction of the "snorkel" needle (Fig. 4). Briefly, the needle portion of a 20- or 22-gauge needle is cut off, shaped to fit the hub of another intact 20- to 22-gauge needle, and soldered in place, leaving the cannula open. The snorkel needle is then attached to a 50- or 100-ml syringe which has been filled with the bacillary suspension to be dispensed. This double needle is then inserted through the rubber stopper of the vial, and, as the culture suspension fills the bottle, excess pressure is released through the snorkel tube. To minimize dangers of aerosol formation, the entire operation is conducted in a negative-pressure safety cabinet. After bottles have been filled, the tops of all rubber stoppers are "sealed" by the application of a thin film of melted paraffin.

To safely remove the thawed bacillary suspension from the bottle, a sterile needle (with attached syringe) is inserted through the rubber stopper and the desired volume is drawn into the syringe. A cotton pledget soaked in 70% alcohol is placed around the vial and the needle to minimize aerosol formation when the needle is withdrawn from the stopper. This suspension then may be diluted for animal inoculation or placed directly onto the desired medium for preparation of a subculture.

DISCUSSION

The preliminary aims of the Trudeau Mycobacterial Culture Collection have been met by the establishment of a routine method of culture preservation and storage. A seed lot of each strain in the collection has been bottled and preserved at −70 C for future use in the preparation of large lots of culture suspension for distribution. In accordance with the observations of Steenken (10) and Steenken and Gardner (11), all virulent strains of *M. tuberculosis* and *M. bovis* are first grown as pellicles on the surface of PB liquid medium buffered at *p*H 7.4. The preparation of the large volume of cell suspension for bottling and distribution is accomplished by grinding the surface pellicle into Middlebrook 7H-9 medium containing ADC enrichment. We attempt to maintain pathogenicity of virulent strains of *M. avium* by selection of the appropriate smooth, transparent colony form (2). In the case of *M. avium* and all other species of mycobacteria, large batches of suspensions for distribution are prepared by growing the cultures in appropriate volumes of enriched 7H-9 broth, harvesting and bottling during the latter stages of logarithmic growth, and storing at

FIG. 3. *Borosilicate glass vials used for storage and shipment of cultures. See text for details.*

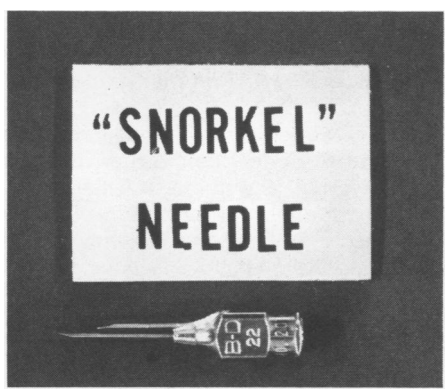

FIG. 4. *"Snorkel" needle used for mass-production bottling of culture suspensions. See text for details of preparation.*

−70 C until mailed. The use of a single, large batch of each organism, coupled with our monitoring of each completed batch, has obviated such fears as clonal selection of populations with divergent metabolic or pathogenic capabilities. The system now has been in use for over 3 years, and routine monitoring of cultures has revealed (i) maintenance of high levels of viability, (ii) reproducibility of taxonomically definitive characteristics, and (iii) persistence of virulence.

Lind has shown (8) that storage of lyophilized BCG at both −25 C and −70 C resulted in essentially 100% survival of organisms for prolonged periods of storage. However, the process of lyophilization resulted in an initial kill of 40 to 50% of the viable bacilli (8, 12). In contrast, our data here reveal a gradual but definite decrease in viable numbers of mycobacteria suspended in various aqueous diluents and stored at −20 C. On the other hand, preparation of liquid suspensions of mycobacteria may be placed in a freezer at −70 C with virtually no loss in viability encountered in the freezing process and maintenance of 100% viability for prolonged periods of storage.

The use of skim milk as a diluent (no. 1) presented two problems. First, it is impossible to obtain a photometric reading to allow a rough assessment of microbial population. Secondly, the rapid thawing of skim-milk suspensions resulted in the production of a mushy coagulum which was difficult to homogenize, and consequently presented problems in plate-counting procedures; this effect was more noticeable in suspensions stored at −20 C than those kept at −70 C.

The lactose diluent (no. 6) proved unsatisfactory for freezer storage because the crystalized sugar was difficult to resolubilize and thus interfered with preparation of uniform suspensions. This apparently has not been a problem when employed with lyophilized suspensions of BCG (A. Frappier, *personal communication*).

For many decades, the Trudeau Mycobacterial Culture Collection, earlier under the capable direction of William Steenken, Jr., has enjoyed a world-wide distribution of cultures. Since institution of the new bottling-preservation system, over 4,000 vials have been sent out (some over 10,000 miles) without a single report of breakage or leakage; we feel this is an excellent testimonial for the glass vials we have employed.

Despite the notorious ability of mycobacteria to clump, we have been exceptionally pleased with the reproducibility of bacterial plate counts over the 3-year period of this study. We are still faced with a dilemma in the preparation of adequately dispersed suspensions of BCG. The method of filtration of BCG suspensions through a membrane filter of 5-μm pore size (4) is excellent for preparation of a single-cell suspension, but in our hands it has been impossible, using membrane filtration, to prepare single-cell suspensions of 10^8 organisms per ml required in some animal experiments. Further work is necessary in this regard. It is also obvious that, unlike *M. tuberculosis* and most other species of mycobacteria (*unpublished data*), BCG strains exhibit a slight loss in viability after 3 years of storage at −70 C. This somewhat alarming observation necessitates a reevaluation of our present methods of preparation for this strain of the species *M. bovis*.

ACKNOWLEDGMENT

This investigation was supported by U.S. Public Health Service contract PH 43-68-640 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Collins, F. M., G. B. Mackaness, V. Montalbine, and M. M. Smith. 1968. A comparison of the Steenken minimal inhibitory concentration and the Canetti proportion methods for determining levels of drug resistance in cultures of *Mycobacterium tuberculosis*. Amer. Rev. Resp. Dis. **98**:189-200.
2. Dunbar, F. P., I. Pejovic, R. Cacciatore, L. Peric-Golia, and E. H. Runyon. 1968. *Mycobacterium intracellulare:* maintenance of pathogenicity in relationship to lyophilization and colony form. Scand. J. Resp. Dis. **49**:153-162.
3. Glover, R. E. 1946. Cold preservation of *M. tuberculosis*. The effects of (a) freeze-drying and (b) low temperature on the viability of *Mycobacterium tuberculosis*. J. Pathol. Bacteriol. **58**:111-114.
4. Grover, A. A., H. K. Kim, E. H. Wiegeshaus, and D. W. Smith. 1967. Host-parasite relationships in experimental airborne tuberculosis. II. Reproducible infection by means of an inoculum preserved at −70 C. J. Bacteriol. **94**:832-835.
5. Gruft, H., M. E. Clark, and M. Osterhout. 1968. Preservation of mycobacterial cultures. Appl. Microbiol. **16**: 355-357.
6. Jones, W. D., Jr. 1957. Simple method for maintaining stock cultures of Mycobacterium species. Amer. J. Clin. Pathol. **27**:363-364.
7. Kubica, G. P., T. H. Kim, and F. P. Dunbar. 1972. *Mycobacterium tuberculosis*, strain H37Rv, proposed neotype of the species. Int. J. Syst. Bacteriol. **22**:99-106.
8. Lind, A. 1967. Stability of dried BCG vaccine stored at −70°, −25° and +4°C. Scand. J. Resp. Dis. **48**:343-347.
9. Mitchison, D. A., A. L. Bhatia, S. Radhakrishna, J. B. Selkon, T. V. Subbaiah, and J. G. Wallace. 1961. The virulence in the guinea pig of tubercle bacilli isolated before treatment from South Indian patients with pulmonary tuberculosis. Bull. W. H. O. **25**:285-312.
10. Steenken, W., Jr., 1935. The influence of the pH on dis-

sociation of B. Friedlander and *M. tuberculosis*. J. Infect. Dis. **56**:273–276.
11. Steenken, W., Jr., and L. U. Gardner. 1943. Vaccinating properties of avirulent dissociates of five different strains of tubercle bacilli. Yale J. Biol. Med. **15**:393–402.
12. Ungar, J. 1949. Viability of freeze dried BCG cultures. Tubercle (London) **30**:2–4.
13. Ungar, J., P. Farmer, and P. W. Muggleton, 1956. Freeze-dried BCG vaccine, methods adopted in preparation of a standard product. Brit. Med. J. **2**:568–576.
14. Wayne, L. G., T. M. Dietz, Ch. Gernez-Rieux, P. A. Jenkins, W. Käppler, G. P. Kubica, J. B. G. Kwapinski, G. Meissner, S. R. Pattyn, E. H. Runyon, K. H. Schröder, V. A. Silcox, A. Tacquet, M. Tsukamura, and E. Wolinsky. 1971. A cooperative numerical analysis of scotochromogenic slowly growing mycobacteria. J. Gen. Microbiol. **66**:255–271.
15. Youmans, G. P., and A. G. Karlson. 1947. Streptomycin sensitivity of tubercle bacilli; studies on recently isolated tubercle bacilli and the development of resistance of Streptomycin *in vitro*. Amer. Rev. Tuberc. **55**: 529–535.