Determination of the Mass of *Mycobacterium leprae* by Electron Microscopy

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The mass of *Mycobacterium leprae*, obtained as a pure suspension from tissues of infected armadillos, was measured electron microscopically using a technique that avoids the need for standards of mass. The mean mass of an individual bacterium was 3.9 ± 1.0 (S.D.) $\times 10^{-14}$ g. Comparative measurements were also made on a small sample of *M. lepraemurium* (whose mass is known). Calculation of the mass of an individual bacterium allows numbers of bacteria in samples to be estimated by direct weighing rather than by counting.

INTRODUCTION

Suspensions of *Mycobacterium leprae* obtained from infected tissues may be quantified by direct counting of stained smears; this has so far been the only method of estimating the total number of bacteria present. The method is tedious, inaccurate and not easily applicable to dried bacteria. Recently, relatively large amounts of purified *M. leprae* have become available from tissues of infected armadillos (World Health Organization, 1976). We have used the electron microscope technique of Valentine (1962) to measure the mass of individual bacteria. Their mean mass may be used to calculate numbers of bacteria in weighed samples of freeze-dried preparations without resort to counting, whilst the range of individual masses may provide information about the extent to which the population is 'damaged' and has lost part of its cytoplasmic contents.

METHODS

Suspensions of bacteria. A suspension of *M. leprae* was prepared from lymph nodes of experimentally infected armadillos by a modification of a published method (World Health Organization, 1976) involving homogenization, treatment with dilute NaOH, digestion with collagenase followed by trypsin plus chymotrypsin, and partition in an aqueous two-polymer phase system (P. Draper, unpublished results). The bacteria were used as a suspension in 0.05% (w/v) Tween 80.

A suspension of *M. lepraemurium* was prepared from livers and spleens of experimentally infected mice by method 2 of Draper (1971). The bacteria were used as a suspension in water.

Electron microscopy. Drops of bacterial suspensions were placed on carbon films supported on 400-mesh copper grids. After 1 to 2 min the liquid was removed from the sides of the grids with filter paper and replaced with a drop of water which was immediately removed in the same way. The grids were allowed to dry in air, then kept under vacuum over NaOH until used.

Bacteria were examined in a Philips EM300 electron microscope operated at 60 kV with a 50 μ m objective aperture and a specimen anti-contamination device. Individual isolated bacteria were photographed at a magnification of 27800 $\times$ on Ilford SP332 film (Ilford Ltd, Ilford, Essex). Exposures were adjusted to give densities of $E < 1.5$ after standard development; this ensured a linear relation between electron exposure and photographic density (Valentine, 1966) as well as being conveniently within the range of the microdensitometer.

Measurements from photographs. Images of bacteria on film were scanned across the width of each

organism at regularly spaced intervals along its length (approx. 20 scans for *M. leprae*, 10 for *M. lepraemurium*), using a double-beam recording microdensitometer (model E12 MkIIIB; Joyce, Loeb & Co., Newcastle upon Tyne). The slit of the instrument was adjusted so that it was small in comparison with the width of the image of the bacteria. Areas under the curves drawn by the instrument were measured by counting squares, and from these mean extinctions (E) for each scan, and thence for each whole bacterium, were calculated.

Areas of images of bacteria were measured from $2\times$ enlarged prints; the outlines were traced on a graphics tablet connected to a computer programmed to calculate areas by a process of strip integration. The calibration of the machine was checked with a planimeter. Lengths and widths of images of bacteria were also measured from the prints.

Theory of mass determination. Measurements of mass with an electron microscope depend on the differential scattering of electrons by regions of the specimen with different mass thicknesses (ρt ; mass per unit area). The electrons scattered by a specimen, and passing within a semi-angle α subtended by the objective aperture at the specimen, give an intensity $I(\rho t)$ at the photographic emulsion. If I_0 is the incident beam intensity, the ratio $I(\rho t)/I_0$ may be used to measure the variation of ρt across the specimen. It is assumed that the density of the photographic image is proportional to the electron exposure. I_0 may be measured using a hole in the carbon support film. In practice both $I(\rho t)$ and I_0 are measured with reference to the support film, but the error in the ratio $I(\rho t)/I_0$ due to this is less than 5%, provided that the support film is less than 10 nm thick, as was the case in our experiments.

The mass thickness can be determined either from an experimental curve of $I(\rho t)/I_0$ against ρt , which requires a series of suitable standard objects (Bahr, 1973), or from a theoretical calculation of $I(\rho t)/I_0$ for a particular objective aperture size and incident beam energy. We have used the latter method, making the assumption that carbon is the main contributor to the scattering of electrons.

The theoretical relationship, to within an error of $\pm 5\%$, is

$$I(\rho t) = I_0 \exp(-S_p \rho t),$$

where S_p is a constant for particular values of α and the electron beam energy. For the values used here (15 mradians and 60 keV respectively), $S_p = 0.0043 \text{ m}^2 \text{ mg}^{-1}$; the units of ρt are then mg m^{-2} . S_p increases with decreasing α and decreasing electron energy. Values of ρt calculated in this way agree with experimental values to within 5%.

The accuracy of the determination of ρt for the bacteria depends on the assumption that they consist of carbon atoms; this is reasonable for an unfixed and unstained biological object consisting mainly of atoms of low atomic number. The minimum value of mass that can be measured depends on the errors in the measurement of $I(\rho t)/I_0$; in our system the smallest detectable mass variation is about $\pm 5 \times 10^{-15} \text{ g}$. The equation relating $I(\rho t)/I_0$ and ρt is valid up to ρt about 200 mg m^{-2} , which is considerably greater than any value found in the present work.

RESULTS AND DISCUSSION

The mean individual mass of a sample of 39 *M. leprae* was $3.9 \pm 1.0 \text{ (S.D.)} \times 10^{-14} \text{ g}$. The mass thicknesses varied over a twofold range; thus for a bacterium of calculated mean area, the mass range was 2.6×10^{-14} to $5.2 \times 10^{-14} \text{ g}$.

The mass of *M. lepraemurium* (sample of 10 bacteria) was $6.4 \pm 1.5 \text{ (S.D.)} \times 10^{-14} \text{ g}$. This agrees well with the value of $6.0 \times 10^{-14} \text{ g}$ obtained by Valentine (1962). The range for a bacterium of mean area was 5.3×10^{-14} to $7.4 \times 10^{-14} \text{ g}$.

The mean length of *M. leprae* was $2.1 \pm 0.5 \text{ (S.D.) } \mu\text{m}$. There was less variation in length than predicted for a population of bacteria growing exponentially and dividing when their length has doubled. In such a population with a mean length of $2.1 \mu\text{m}$, the theoretical standard deviation is 0.6 (B. Hammond, personal communication). The mean width was $0.45 \mu\text{m}$. In thin sections the width of these bacteria is 0.25 to $0.30 \mu\text{m}$, suggesting that they were distorted in shape by drying without fixation. It would be necessary to examine freeze-dried or critical-point dried bacteria to obtain a 'true' width. Distortion would not affect the accuracy of the mass measurements.

The images of *M. lepraemurium* were more uniform than those of *M. leprae*. An objective measure of the difference was given by the standard deviation of the mean extinction of each bacterial image, calculated from the individual scans (20 each for *M. leprae*, 10 for *M. lepraemurium*). The deviations for *M. lepraemurium* averaged 0.038 and ranged from 0.024 to 0.067 while values for *M. leprae* were 0.054 and 0.019 to 0.113 respectively.

Irregular staining in the light microscope, which corresponds to an irregular image in the electron microscope, indicates loss of viability (Rees, Valentine & Wong, 1960). The irregularly stained bacteria are thought to have lost part of their contents. We found no correlation between ρt for individual bacteria and the standard deviations of the mean extinctions, although such a correlation should exist if the population consisted of a mixture of viable (even, heavy) and degenerate (irregular, light) bacteria. We conclude that either the isolation process makes all the bacteria 'degenerate' or that it alters them in some way so that the bacterial cytoplasm collapses irregularly on drying in a vacuum. *Mycobacterium leprae* had a range of mass that overlapped that of *M. lepraemurium*, which is about the same size and usually highly viable in mouse tissues. We conclude that at least some of the *M. leprae* measured contained most of their original cytoplasmic contents. Light microscope measurements show that *M. leprae* from armadillos may contain up to 20% of 'intact' bacteria (R. J. W. Rees, personal communication). There is immunological evidence (M. Harboe, personal communication) that the rather drastic techniques used to isolate the bacteria cause little damage to them.

Apart from the work of Valentine (1962) there have been no attempts to weigh mycobacteria directly. Larger bacteria may be more conveniently weighed by interference microscopy (Mitchison, 1961). Mycobacteria such as *M. leprae* and *M. lepraemurium* have widths close to the limit of resolution of the light microscope, so that the interference method cannot be very precise. M. R. Young (personal communication) obtained a value of 5×10^{-14} g for a preparation of *M. leprae* similar to the one used here.

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