Growth (without Multiplication) of *Mycobacterium lepraemurium* in Cell-free Medium

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SUMMARY

Leprosy bacilli have not been cultivated with certainty in any cell-free medium, but the medium now described consistently supported considerable elongation of the organisms of murine leprosy (*Mycobacterium lepraemurium*), though without evidence of multiplication. The mean length of the bacilli doubled in about the generation time obtaining in host cells (7–14 days) and quadrupled before the bacilli became degenerate after about 2 months. The obligate acidity and other factors concerned in this growth were investigated, and are discussed in relation to the problem of the extracellular cultivation of leprosy bacilli.

INTRODUCTION

In the present context, 'growth' of a bacterium is defined as a gradual 'increase in bulk by the process of organic life', without this necessarily being accompanied by multiplication. (The distinction is illustrated by Nicolay (1585): 'Great bushes, and wilde brambles, which in process of time... were so grown and multiplied'.) A cell-free medium is defined as one without intact tissue cells.

Continuous intracellular multiplication of *Mycobacterium lepraemurium*, the rat leprosy bacillus (used often as a laboratory model in leprosy studies), has recently been obtained in cultures of rat fibroblasts (Rees & Garbutt, 1962), but this species has never been shown unequivocally to multiply at all in any cell-free nutrient medium (Eddy, 1937; Gray, 1952). We have similarly failed with many such media, and have moreover found with the electron microscope that most of the bacilli are degenerate (dead) within 2–3 weeks at 37°. However, by adding sucrose to a concentration of 10% to a liquid medium (modified from that of Dubos & Davis, 1946) that is used in these laboratories for subsurface culture of *Mycobacterium tuberculosis*, Hart & Valentine (1960) obtained an increase in bacillary length in *M. lepraemurium* and a delay in its degeneration. We now report the further development of this work, which provides evidence of a living process, even though no multiplication has been detected.

METHODS

Source of the bacilli

The bacilli were provided by Dr R. J. W. Rees and his co-workers:

(a) *From infected mice.* The liver of a mouse of the albino P strain which had been inoculated intravenously 4–6 months previously with *Mycobacterium lepraemurium* (Douglas strain), and which had usually received suramin to enhance the infection,

was homogenized (see Hart, Rees & Valentine, 1962*a*), and the bacilli partly freed from the tissue components by the method of Garbutt, Rees & Barr (1962) and then suspended in physiological saline solution containing 1% albumin (bovine plasma fraction V, Armour), to give about 2×10^9 acid-fast bacilli/ml., as counted by the method of Hart & Rees (1960). The organisms were well dispersed and predominantly single.

(b) *From infected tissue cultures.* A suspension, in Hanks balanced salt solution, of rat fibroblasts containing bacilli of *M. lepraemurium* (Garbutt *et al.* 1962) was centrifuged at 1200 rev./min. (250 g) for 5 min. to deposit the tissue cells, which were concentrated by removing an appropriate amount of the supernatant fluid. In some experiments the bacilli were released from the cells by exposure to ultrasonic vibration (400 kc./sec. supplied by a 500 W. generator) for 1 min. In the present work tissue culture-grown bacilli were used much less frequently than bacilli from infected mouse livers. Unless otherwise stated, the infected mouse liver was the source for inoculation into cell-free medium.

Table 1. *Final concentrations of ingredients of the present medium for studying elongation of Mycobacterium lepraemurium bacilli*

Ingredient	% (w/v)
Casamino acids (Difco)	1.6
Asparagine	0.75
Na ₂ HPO ₄	0.14
KH ₂ PO ₄	0.06
Sodium citrate	0.08
MgSO ₄ ·7H ₂ O	0.03
Glycerol	2.9
Albumin	0.18*
Sucrose	7.4

* 0.15% when the inoculum was from tissue culture.

Present medium

The basal medium was composed of: Difco (Bacto) 'Casamino acids' (certified), 29 g.; *L*-asparagine monohydrate, 13.5 g.; anhydrous Na₂HPO₄, 2.5 g.; KH₂PO₄, 1.0 g.; trisodium citrate, 1.5 g.; MgSO₄·7H₂O, 0.6 g.; glycerol, 53 ml.; de-ionized water to 1000 ml.; adjusted with HCl or NaOH to pH 6.2 or as required; autoclaved at 115° for 20 min. This autoclaved solution was distributed in 4.5 ml. amounts into 1 oz. (28 ml.) screw-capped bottles, to which were added 0.25 ml. of 5% aqueous solution of albumin (fraction V) sterilized by filtration; 1.2 ml. of autoclaved 50% (w/v) sucrose solution; solutions of any test substances; water to 7.9 ml.; 0.2 ml. of the bacillary suspension from mouse liver homogenate or tissue culture, giving a final total volume of 8.1 ml., containing about 5×10^7 , or 10^6 – 10^7 , bacilli/ml., respectively. The final concentrations of the ingredients in the complete medium are shown in Table 1; in addition a small amount of liver or fibroblast tissue from the inoculum was inevitably present.

Assessment of growth of bacilli in medium

A bacillary suspension in the medium was immediately fixed by adding formaldehyde to a concentration of 1%; this served as a sample of the inoculum (for a base

line) and was stored at 4° (no significant change in the length of the bacilli occurred during storage). Other suspensions in the same medium were incubated, at 37° unless otherwise stated, for periods up to 2 months and were then similarly fixed. The inoculum sample and the incubated suspensions were diluted 1/8 in water (to decrease the sucrose concentration) and then centrifuged at 2000 *g* for 30 min. in 8 ml. Pyrex tubes. The deposited bacilli were dispersed in about 0.1 ml. of supernatant fluid by exposure to ultrasonic vibration (400 kc./sec.) for 1 min.

Samples of the deposits were examined in the electron microscope, adjusted to a magnification of 10,000 by means of standard spheres (diam. 0.26 μ) of polystyrene latex. A series of concentric circles, the circumferences of which were spaced by 0.5 cm., drawn on the fluorescent screen on which the final image was seen, allowed the lengths of the bacilli to be estimated to the nearest 0.1 μ . The lengths of 100 or more bacilli from each sample were measured in this way, and the frequency distributions of lengths and their means calculated. At the same time the proportion of bacilli which appeared degenerate (McFadzean & Valentine, 1959) was estimated. In addition, the bacillary deposits were stained by the Ziehl-Neelsen method and examined with the light microscope; a grading of length was made without formal measurement, and, using the modified stain described by Rees & Valentine (1962), the presence of degenerate bacilli was assessed. (Unless otherwise stated, bacillary lengths and proportions degenerate refer to the results obtained with the electron microscope.)

RESULTS

Optimal conditions for elongation of the bacilli of Mycobacterium lepraemurium

The nutrient medium used by Hart & Valentine (1960) required a high concentration of sucrose (or glucose) in order to obtain substantial lengthening of the bacilli. However, the results were irregular. They were somewhat better when Fe³⁺ was added to 15 μ g./ml. and Mg²⁺ was increased to 14 mM (Hart, Rees & Valentine, 1962*b*); but these additions were not necessary with the present medium. In the latter, the Casamino acids were increased 6 and the asparagine 25 times; the sucrose was 7.4 %; and the solution had a specified acidity (see below). Elongation was then more rapid, more uniformly distributed in the bacterial population, and reached a higher final figure; and the results were regularly reproducible.

The acidity of the medium was critical for elongation to occur. Figure 1 shows the mean length and the degree of degeneration of the bacilli, obtained from infected mouse liver, after 17 days of incubation at 37° in the medium at different initial pH values (the pH value remained stable during this period). The greatest elongation (mean length nearly 4 μ) occurred around pH 6.0-6.4, with a rather sharp peak; and there was virtually none (mean length about 2 μ compared with 1.8 μ in the original inoculum) at pH 7.2 and more, or at pH 5.0 and less. The curve for the proportion of bacilli which were degenerate (assessed by electron microscopy) was nearly a mirror-image of that for lengthening; the majority were degenerate after incubation above pH 7.2 and below pH 5.5, whereas over 80 % (compared with over 90 % in the original inoculum) were still not degenerate at pH 6.4. It has not been possible to separate the association between acidity and lengthening and that between acidity and decreased degeneration; the optimum pH value was about the same for both and, moreover, all alterations so far made to the medium which

resulted in abolition of the elongation at pH 6.2 were accompanied also by degeneration similar to that seen at neutrality.

On the basis of these findings, a value of about pH 6.2 was specified for the medium when elongation was desired, and of about pH 7.2 when it was not. The progress, in a typical experiment, of elongation and degeneration of the bacilli (from infected

Fig. 1. The lengths (○) and proportions degenerate (▲) of *Mycobacterium lepraemurium* organisms after incubation at 37° for 17 days in the medium adjusted with HCl or NaOH to different pH values. The mean length of the bacilli in the inoculum was 1.8 μ and less than 5% were degenerate.

Fig. 2. The lengths (○) and proportions degenerate (▲) of *Mycobacterium lepraemurium* organisms after different periods of incubation at 37° in the medium at pH 6.0.

mouse liver) during 2 months of incubation at 37° in the medium at pH 6.0 is shown in Fig. 2. Some lengthening was evident by 2 days. The mean length of 1.9 μ in the original inoculum doubled by 7 days; after between 7 and 14 days the rate of increase gradually decreased, and the mean length at 60 days was 7.1 μ . The proportion of organisms which were degenerate (initially less than 5 %) was still less than 10 % at 7–14 days; it then increased gradually, reaching 35 % by 30 days and 70 % by 60 days, by which time elongation had virtually ceased. Hence degeneration, already marked at 2 weeks at pH values unfavourable to elongation (Fig. 1), was postponed but not avoided under these optimum conditions.

The frequency distribution of lengths of bacilli in the original inoculum is compared (Table 2) with that after incubation for 17 days in the medium. Initially, only 4 % of bacilli measured more than 3 μ and 32 % more than 2 μ ; after the incubation the corresponding percentages were 62 % and 87 %. The increase in mean length is clearly due to a general shift and not merely to a few very long bacilli.

Table 2. *Length distribution of Mycobacterium lepraemurium organisms before and after incubation for 17 days at 37° in the medium at pH 6.0*

Length (μ)	% bacilli	
	Initially	After 17 days
0–1	0	0
1–2	68	13
2–3	28	25
3–4	4	25
4–5	0	17
5–6	0	8
6–7	0	4
7–8	0	4
8–9	0	2
9–10	0	2

The necessary constituents of the medium were investigated further, first, by adding albumin and sucrose in the same concentrations to phosphate buffer pH 7.3 and 6.2, the phosphate being finally M/50 and M/125 (that in the medium was M/70). After incubation for 17 days in these four buffer mixtures, there was no appreciable increase in average length of the bacilli at either pH value, whereas the complete medium showed none also at the higher pH value, but more than trebling of initial mean length at the lower pH value. The proportion of organisms which were degenerate was 5 % in the inoculum, 60 % or more in all the four buffer mixtures after incubation for 17 days, as compared with only 10 % at pH 6.2 in the complete medium. Next, to a similar buffer mixture at pH 6.2 (phosphate M/70; albumin and sucrose as before) glycerol was added to 2.9 % (w/v), as in the complete medium. Some elongation was now evident after incubation (though less than in the complete medium), whereas there was again none in the buffer mixture without glycerol.

The microscopic appearances of *Mycobacterium lepraemurium* in the conditions described are illustrated. Plate 1 shows, by electron microscopy: a normal bacillus from infected mouse liver (Pl. 1, fig. 1); a degenerate bacillus such as is produced by incubation for 2 or more weeks in phosphate buffer, or in the complete medium at pH 7.2 (Pl. 1, fig. 2); a long apparently healthy bacillus as produced in the

medium at pH 6.2 (Pl. 1, fig. 3); a long bacillus which has eventually degenerated (Pl. 1, fig. 4). Plate 2 shows, by light microscopy: bacilli from mouse liver immediately after inoculation into the medium (Pl. 2, fig. 5), and also the bacilli after incubation for 17 days at pH 6.2 (Pl. 2, fig. 6) and at pH 7.4 (Pl. 2, fig. 7); bacilli in tissue culture cells as inoculated into the medium (Pl. 2, fig. 8), and their appearances after incubation for 14 days at pH 6.1 (Pl. 2, figs. 9, 10). In the light microscope, long bacilli in the early stages showed a characteristic beaded appearance of the stained material which made assessment of degree of degeneration more difficult than in the electron microscope; degeneration in short bacilli, as well as in long bacilli at a later stage, was easier to assess from the gross irregularity of the stained material.

Increase in weight of the bacilli

The dry weights of the bacilli could be estimated from the electron micrographs by using electron-scattering theory (Valentine, 1962). In a typical experiment, the mean dry weights so obtained before and after 2 weeks of incubation in the medium at acid reaction were: bacillus inoculated into the medium = 5.9×10^{-11} mg.; bacillus after incubation for 14 days in the medium = 12.0×10^{-11} mg. During this 14-day period the mean length of the bacilli increased from 2.1 to 4.6μ , i.e. almost in the same proportion as the weight. In the same time there was usually a slight (c. 20 %) increase in the width of the bacilli.

The effect of unfavourable environment on subsequent ability to elongate

The bacilli were incubated in the medium for various periods at a pH value either too large or too small for elongation to occur; the pH value was then changed to the optimum pH range by adding acid or alkali. Thus, after 1 day at pH 7.7, followed by 2 weeks at pH 6.0, the bacilli lengthened moderately well, whereas when this change in pH value was made (from pH 7.7 or 7.2 to pH 6.0) after 1 week, they lengthened little or not at all. Similar results were obtained when 1 week at pH 4.4 or 4.8 was followed by 2 weeks at pH 6.3. On the other hand, organisms incubated in the medium at pH 6.2 for 1 week, and then transferred to fresh medium at pH 6.2 and 7.0 for a further 2 weeks, continued to lengthen at the lower pH value but not at the higher. Finally, bacilli which were incubated in phosphate buffer mixtures (see above) for 2 weeks, whether at pH 6.2 or 7.3, and then (when more than half were seen to be degenerate by microscopy) transferred to the complete medium at pH 6.2, showed no subsequent elongation. It may be concluded that, when the pH value or the composition of the medium was unfavourable to elongation, not only did degeneration (as seen by microscopy) rapidly ensue (see above), but the ability to lengthen when subsequently the pH value or the composition of the medium was made favourable was soon diminished and abolished.

The natural resistance of *Mycobacterium lepraemurium* to killing by alkali was tested by exposing a bacillary suspension (from infected mouse liver) in 0.5 or 0.05 % albumin saline to 0.1 N or 0.02 N-NaOH for 30 min. at 35°, and washing the organisms, before inoculating them into the medium at pH 6.2. Resistance to mineral acid was tested by exposing a suspension in 0.025 % albumin saline to 0.1 N-HCl for 2 hr. at 37°, neutralizing, and then inoculating into the medium at pH 6.2. In both cases the capacity for lengthening was still evident, though

diminished. In this resistance, more particularly to acid, *M. lepraemurium* resembles *M. tuberculosis*, in which species this property is well known.

The effect of temperature

Bacilli were kept in medium at 42°, 37°, 34°, 24° and 4° for a month. The elongation at 34° was slightly less than at 37°, and there was none at the other temperatures. In another experiment samples of a bacillary suspension from infected mouse liver in the usual 1 % albumin saline were kept at 37° and 4° for 14 days, samples being withdrawn periodically from each lot of suspension and inoculated into the medium at pH 6.0, which was then incubated for 14 days at 37° and the bacilli examined for lengthening. Bacilli sampled from the 37° suspension on days 0 and 2 showed lengthening to a similar and considerable degree in the medium, but those sampled at day 5 lengthened hardly at all. In contrast, bacilli from the 4° suspension showed considerable lengthening when sampled up to day 8, and a little when sampled on day 14.

Tissue contamination

Mycobacterium lepraemurium is intracellular *in vivo*, but the method of preparation of the bacillary suspensions from mouse liver breaks up the tissue cells. Thus electron and light microscopy have not revealed any intact liver cells in samples of bacilli examined either before or after incubation in the medium for 14 days. It was, nevertheless, conceivable that some few liver cells were necessary for elongation to occur. This was excluded by the finding that lengthening could occur after treatment with NaOH (p. 48), which decreased the total cold trichloroacetic acid-precipitable material of the liver in the final bacillary suspension by 80–90 %. Such digestion not only would have destroyed any whole cells but also would have removed most of the remaining cell fragments.

Towards further exclusion of an essential role of tissue cells, a suspension of *Mycobacterium lepraemurium* was specially freed from liver remnants by additional differential centrifugation, and then tested for bacillary elongation in the usual manner; this was found to be similar to that of a normal preparation of bacilli. With a similar object, bacilli from a tissue culture were released from the fibroblasts by ultrasonic vibration, and the suspension washed twice and resuspended in 1 % albumin saline; again lengthening occurred.

Although it seems unlikely, therefore, that contaminating tissue played an essential part in the bacillary growth in the complete medium, it is possible that the residual liver in the preparations from mice contributed some of the nutritional requirements for growth.

Stale medium

Bacilli were incubated for 2 weeks in the complete medium at pH 6.2; the suspension was then centrifuged and fresh bacilli inoculated into the supernatant fluid, and incubation carried on for a further 2 weeks. In another experiment a fresh suspension was inoculated into the supernatant fluid from some of our earlier medium which had been in contact at 37° with bacilli for 2 months. In both experiments the fresh bacilli lengthened in the old supernatant fluid. 'Staling' of the medium appeared therefore not to be the cause of failure to multiply, nor of the eventual degeneration of lengthening bacilli.

Alterations to the medium

Many additions were made to the present medium in the hope of obtaining division and multiplication of the bacilli, or a further delay in their degeneration, but these additions, among which was mycobactin (the growth factor for *Mycobacterium johnei*), were ineffective both at neutrality and in the acid pH range optimal for lengthening. Substitution of other carbon sources for sucrose, in high or low concentrations (e.g. glucose (Hart & Valentine, 1960), sorbose or erythritol) gave no advantage. Omission of the sucrose altogether usually decreased the degree of elongation, though not much, but the results were then less regular and uniform. Omission of glycerol led to a much more pronounced diminution in elongation. On the other hand, some elongation (at pH 6.2) still occurred (both with well-washed tissue-culture bacilli and with liver-contaminated bacilli from mice) when the asparagine and Casamino acids were omitted, leaving salts, glycerol, albumin, sucrose, i.e. a mixture rather like the buffer mixture with glycerol (p. 47) and giving similar results.

Results in other media

A few conventional media were tested for their capacity to promote the lengthening of *Mycobacterium lepraemurium*. These included Hedley Wright broth, glycerinated broth, and Hanks's basal salt solution with human cord serum. At about pH 7 and 6, and both with and without sucrose, the bacilli degenerated within a few weeks without obvious lengthening.

The effect of isoniazid

As is well known, isoniazid suppresses the *in vitro* multiplication of *Mycobacterium tuberculosis*; this substance at 1 µg./ml. also completely inhibited the elongation of *M. lepraemurium* at pH 6.0–6.8. On the other hand, a substrain obtained from an infected mouse and which showed isoniazid resistance *in vivo* required 25 µg. isoniazid/ml. for complete inhibition (Hart *et al.* 1962*b*).

Total count of acid-fast bacilli

The possibility of significant changes in the total population of acid-fast bacilli was examined by Dr R. J. W. Rees (method of Hart & Rees, 1960). The counts were made immediately after inoculation into the medium and also after incubation for 17 days at pH 7.0 and at pH 6.0. About 80 bacilli were counted in each case, sufficient to detect almost certainly ($P = 0.001$) a change of 50 % or more in the population. In fact, the counts diminished slightly from 1.7×10^7 /ml. initially to 1.5×10^7 /ml. (pH 7.0) and 1.4×10^7 /ml. (pH 6.0) after 17 days, but the differences were not significant ($P > 0.15$). Almost certainly, therefore, any increase or decrease in the population during the period of maximum bacillary elongation was less than 50 % and consequently unimportant or non-existent.

DISCUSSION

Gross elongation of the bacilli of many bacterial species *in vitro* has been frequently described, the common factor being a change in the physical or chemical environment unfavourable enough to prevent multiplication (division) while still permitting

growth. Mycobacteria are no exception, e.g. *Mycobacterium johnei* in culture medium containing minimal concentrations of its growth factor mycobactin (Hart, 1958) and *M. tuberculosis* in medium made toxic by urea (Hart, unpublished) or when incubated in certain buffer mixtures (see below). There would be little point in describing elongation in *M. lepraemurium* in a cell-free medium were it not that multiplication has not been achieved for this species in any such medium. Consequently, growth in the mass and length of *M. lepraemurium* bacilli may be an advance towards the possibility of obtaining complete *in vitro* cultivation; and, unlike the case with other bacteria, the physical and chemical conditions required for maximum elongation can be a guide to those most favourable for this organism.

That the elongation described represents an active vital process and not a passive stretching, or a mere accumulation of material from the medium, is supported by the present evidence—in particular, the gradual progress to a doubling of length in about the time required for one generation cycle in living mice or rats or in fibroblast cultures, namely 7–14 days (Hilson & Elek, 1957; Rees & Garbutt, 1962), the suppression of the process by low concentrations of isoniazid, and the failure of isoniazid to do so with a substrain which was isoniazid-resistant *in vivo* (Hart *et al.* 1962*b*).

Although the bacillary preparations from mice contained some liver material, the ability of the bacteria to lengthen after treatment with sodium hydroxide (which would have digested any whole liver cells still present and removed most of the cell fragments) appears to exclude any analogy with the cell-micro-organism association of tissue culture. On the other hand, the small amounts of residual tissue may have made a nutritional contribution towards the growth.

Elongation of *Mycobacterium lepraemurium* can be observed as a normal process within the host cells in the initial phase of slow multiplication in progressive murine leprosy infection in mice (Hilson & Elek, 1957; Chang, 1960; Hart *et al.* 1962*a*) and in monocytes maintained *in vitro* (Chang, 1961). We have noted abnormal elongation in a minority of the population of the slow-growing *M. johnei* in cultures in or near foci of multiplying short bacilli. However, the extracellular lengthening which we can now obtain with *M. lepraemurium* is far more striking, in that almost all the bacilli are affected, most of them finally reaching three to five times their initial lengths. This abnormal elongation, terminated eventually by degeneration, is presumably a consequence of inability to divide. We have observed a possibly analogous, though less marked, elongation without obvious multiplication in *M. tuberculosis* incubated for several weeks at 37° in phosphate buffer (pH 6.0) containing albumin and sucrose (a mixture which had given some lengthening of *M. lepraemurium* if glycerol was added, see p. 47); the *M. tuberculosis* bacilli remained short in buffer alone.

The present medium was developed from the Dubos-type formula (without Tween 80), but has a much higher content of amino acids, a high concentration of sucrose, and an acid pH value (about pH 6.2). None of the limited number of other media so far tested has given similar results either at neutral or at acid pH values, with or without sucrose. On the other hand the sucrose, while beneficial in the present medium (optimum about 10%; Hart & Valentine, 1960), is not essential for the bacillary elongation; consequently the mode of its action in such high concentration is perhaps not of particular interest. Of the other constituents of the

present medium, glycerol appears to be important for obtaining elongation, but the role of the asparagine and Casamino acids is less certain, since their omission still allowed some lengthening to occur with organisms from infected mouse liver and also with well-washed tissue-culture bacilli. However, the relatively small amount of nitrogen required for growth could have been provided under these conditions by remnants of liver cells or tissue-culture fibroblasts in the preparations, by the albumin, by the autolysis of bacterial cells, or even from the air as has been suggested to explain a multiplication of *Mycobacterium tuberculosis* in a 'nitrogen-free' medium (Hedgecock & Costello, 1962).

The narrow range of pH values for optimum elongation (pH 6.0–6.4) was less surprising than the virtual absence of elongation at about pH 7.2. For, although the optimum range for multiplication of *Mycobacterium tuberculosis* has been reported to be in the range pH 6.0–6.5 (Bekierkunst, 1957; Hedgecock & Costello, 1962), both this species and the more exacting *M. johnei* give profuse yields when cultivated at neutrality; moreover, the elongation of *M. tuberculosis*, mentioned above, was observed at pH 7.0, also.

The only method of testing chemotherapeutic agents in a murine leprosy infection is *in vivo*, or, latterly, in tissue culture. Complete inhibition of elongation of bacilli of *Mycobacterium lepraemurium* by isoniazid suggests that the doubling or more of mean length which occurs in the first 1–2 weeks of incubation in the present medium without the drug, and which is easily appreciated in the light microscope, could be used as a means for a preliminary assessment *in vitro* of the activity of potentially antileprosy agents for use in man; this possibility is being investigated. However, it does not necessarily follow that this bacillary elongation, since it is not accompanied by division, can provide information relevant to activity against a progressive infection in the animal body.

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EXPLANATION OF PLATES

PLATE 1

Electron microscope pictures of *Mycobacterium lepraemurium* before and after incubation ($\times 27,000$).

Fig. 1. A normal bacillus from infected mouse liver.

Fig. 2. A bacillus after incubation for 28 days in phosphate buffer. A typical degenerate appearance.

Fig. 3. A bacillus after incubation for 14 days in the medium at pH 6.2. It has lengthened without yet showing a degenerative appearance.

Fig. 4. A bacillus after lengthening, followed eventually by an appearance typical of degeneration.

PLATE 2

Light microscope pictures (Ziehl-Neelsen stain) of *Mycobacterium lepraemurium* before and after incubation in the medium ($\times 1900$).

Fig. 5. Normal bacilli obtained from infected mouse liver. The organisms are well dispersed and predominantly single. They are homogeneously stained.

Fig. 6. Similar bacilli after incubation for 17 days at pH 6.2. They are long and many have a beaded appearance.

Fig. 7. Similar bacilli after incubation for 17 days at pH 7.4. They have not lengthened and many are unevenly stained.

Fig. 8. Normal bacilli in tissue culture fibroblasts.

Figs. 9, 10. Similar bacilli after incubation for 14 days at pH 6.1. The fibroblasts have largely autolysed, the organisms are long and some appear beaded.