Cultivation of *M. leprae* including leads from *M. lepraemurium*

Chairman: R. S. Weiser

Further Analysis of the Growth (Elongation) Phenomenon of *Mycobacterium lepraemurium in vitro*, and Relevant Studies with *Mycobacterium leprae*

P. D'Arcy Hart, F.R.C.P.[1]

Many bacilli elongate when in an unfavorable environment, but on restoration of normal conditions cell division and multiplication can occur and the long forms become replaced. On the contrary, it is still doubtful whether *Mycobacterium lepraemurium* has been observed to multiply in a cell-free medium; consequently elongation, even though an irreversible process apparently, may be considered—at least for the moment—an advance.

[1]National Institute for Medical Research, Mill Hill, London N.W.7, England.

Many of the features of elongation of *M. lepraemurium* in a cell-free medium have already been reported ([3]), and these will only be summarized here. The usual source of bacilli has been homogenized infected mouse liver; the inoculated liquid medium is incubated for several weeks at 37°C and examined by Ziehl-Neelsen stain, or the electron microscope, for length and degeneration. Lengthening is apparent by 2 days, with doubling usually by 7-14 days; the rate of increase gradually declines, a maximum of about 3-4 times the initial

length being reached at about 2 months, when nearly all the bacilli have become degenerate. Total counts (by R. J. W. Rees) have shown no significant changes in numbers.

The medium is fairly specific. It was modified from a Dubos-type formula, but has high concentrations of amino acids and sucrose (7.5 per cent). A striking feature is the critical range of the pH. The optimum is at 6.0-6.5; at pH 7.2 elongation is absent and degeneration occurs early. Various other liquid media have failed to show elongation, even at low pH. On our medium, acidified and solidified with agar, elongation also takes place, but more irregularly than in the liquid medium.

Evidence that elongation of *M. lepraemurium* is a vital process (even of a low order), rather than a passive stretching or a mere accumulation of material, is provided by the following observations: (1) the bacterial dry weight (as estimated from the electron micrographs) increases in proportion to the length; (2) susceptibility to temperature of incubation is as might be expected for bacterial growth; (3) isoniazid suppresses elongation, failing, however, to do so in the case of an isoniazid-resistant substrain (as a matter of interest, streptomycin and diaminodiphenylsulfone have little effect, while the phenazine B.663 inhibits).

Recent work has followed a number of directions. The possibility that contaminating liver fragments provide a relevant nutrient or cellular adjuvant appears unlikely, because elongation by *M. lepraemurium* occurs equally well when the bacilli used had been released from tissue-culture fibroblasts or were obtained from an infected foot pad (R. J. W. Rees), and because, moreover, after sodium hydroxide digestion of a liver-derived bacillary suspension, or after purification by passage through a sucrose gradient until the bacilli were virtually freed from tissue debris, elongation was still shown.

Several strains of mycobacteria isolated by K. R. Chatterjee from mice inoculated with human leprosy material were tested; they gave good elongation under conditions similar to those used for *M. lepraemurium;* their drug susceptibility was also similar. In sharp contrast, bacilli both direct from human leprosy material and from a foot pad infection following inoculation of such material have so far consistently failed to elongate either in liquid or in solid medium, at either acid or neutral pH. It would be interesting biologically, and might prove useful diagnostically if *M. leprae* should be unable to elongate under conditions successful for *M. lepraemurium.*

Elongation takes place at a stage in successful tissue culture of *M. lepraemurium* ([2]); but the present phenomenon, with its increase in cell substance but ultimate degenerative course, seems to be a sort of "dead-alive" process, and possibly an unusual or even unique one in bacterial species. It was of interest to attempt to clarify the metabolic processes involved. Two approaches to this problem have been made.

One approach (in collaboration with M. R. Young) is to examine the structure of *M. lepraemurium* (in fresh suspension) under ultraviolet light at a wave length of 257 $\mu\mu$, close to that specific for nucleic acids, before and after elongation. This procedure (Figs. 1 and 2) reveals polar bodies in the short bacilli and granules along their length. These granules appear to increase in number in proportion to the increase in length after elongation. It is tempting to ascribe this increase in granules to an actual increase in DNA and/or RNA, but (see below) it could equally well be due to an increase in other cell constituents (e.g., polyphosphates) carrying nucleic acids without necessarily an increase in the latter.

Because the ultraviolet light absorption cannot distinguish between DNA and RNA, we had recourse (also with M. R. Young) to the fluorochrome technic, adapted from that described by Anderson, Armstrong and Niven ([1]), but using Euchrysine ([4]) on fresh, unfixed suspensions of *M. lepraemurium* at pH 7.4. Under these conditions the dye shows virtually no entry in *M. lepraemurium* before elongation (unlike *M. phlei, M. tuberculosis* and *M. johnei*), but enters fully after elongation, perhaps because of damage of the

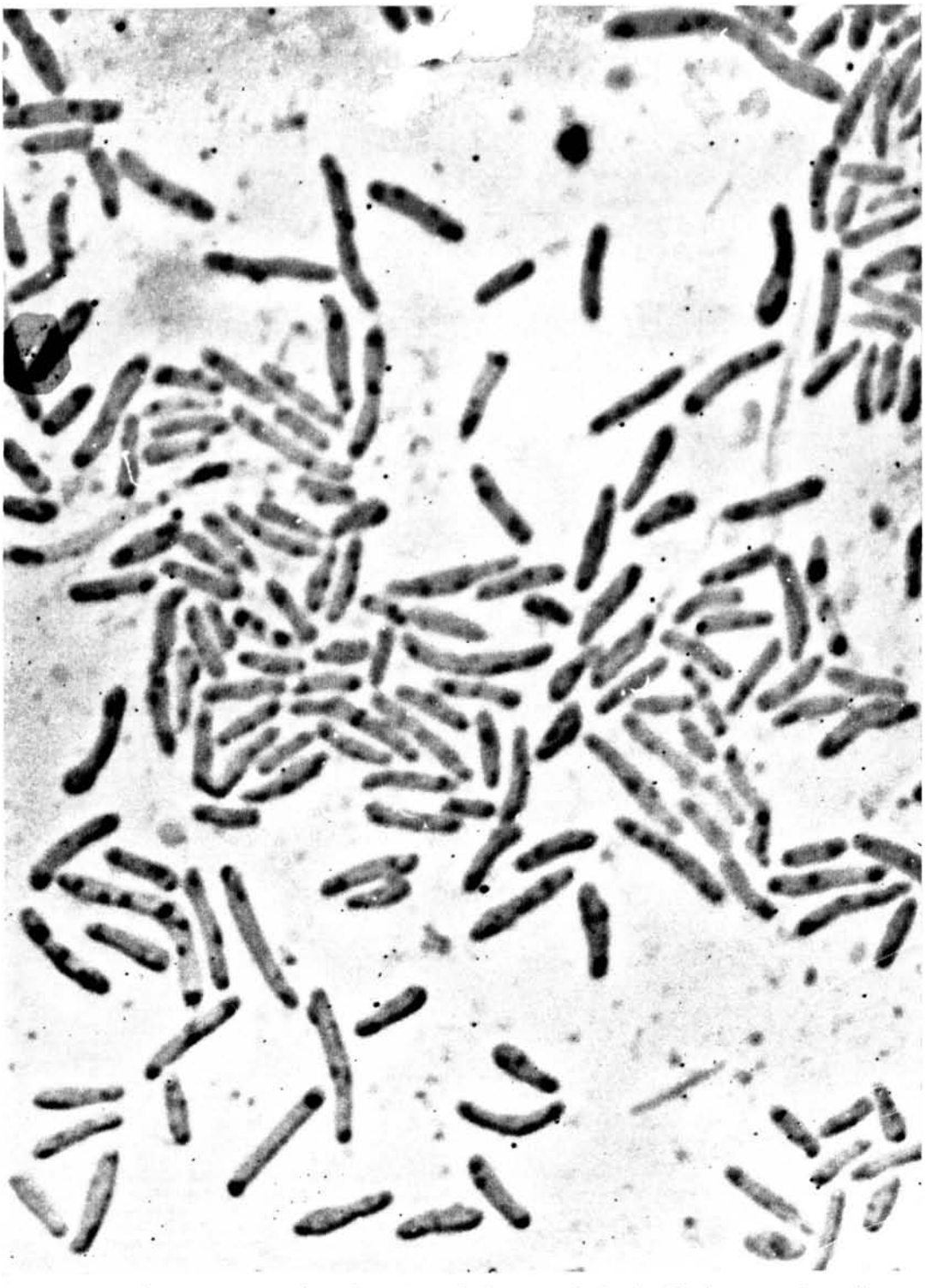

FIG. 1. *M. lepraemurium*, short form (i.e., before incubation). Fresh suspension taken at 257 mμ. ×8,500.

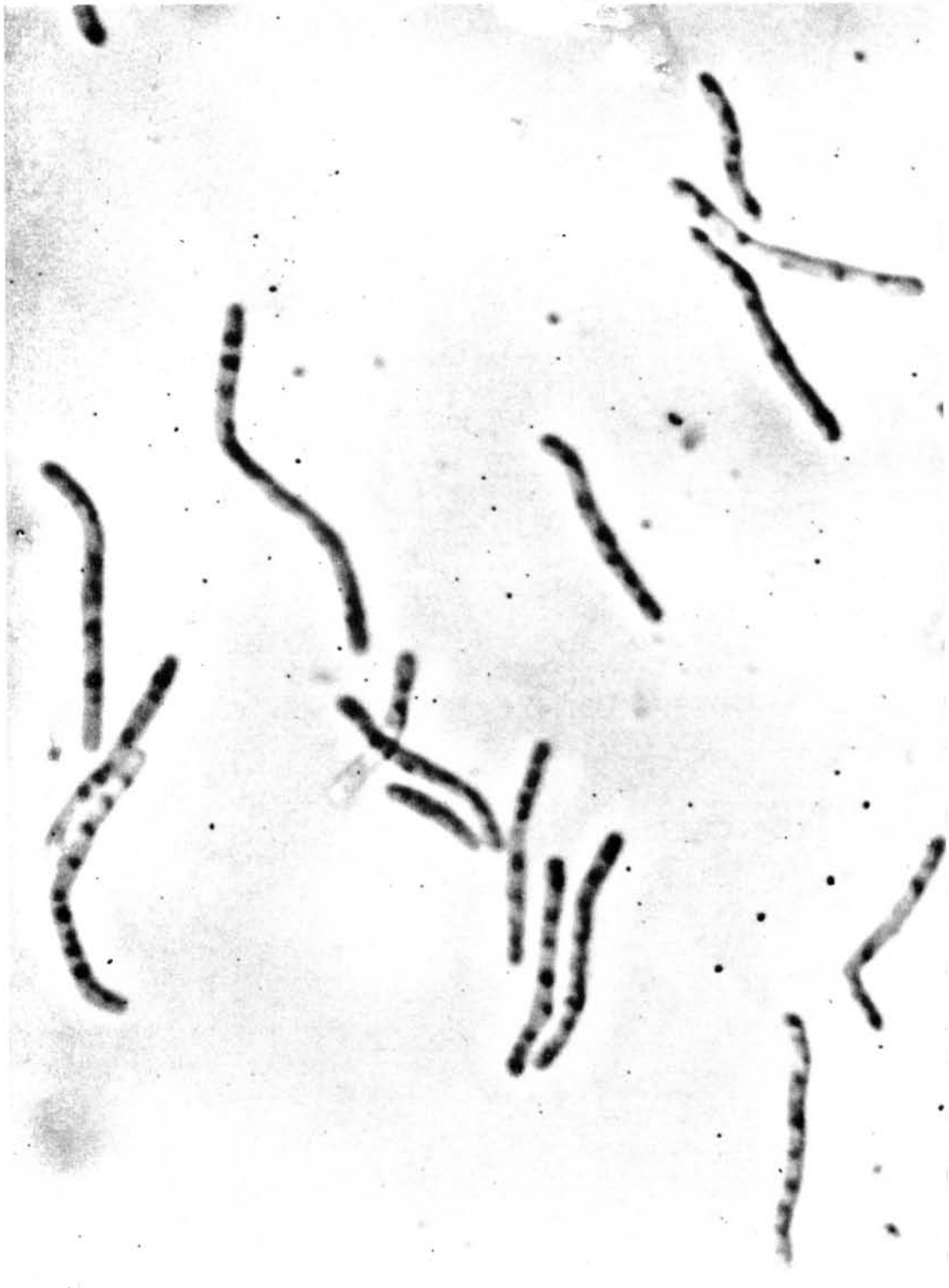

FIG. 2. *M. lepraemurium*, long form (i.e., 2 weeks at 37°C in medium at pH 6.5). Fresh suspension taken at 257 mμ. ×8,500.

cytoplasmic membrane, with resulting reduction of the normal relative permeability of this microorganism. In the elongated bacilli the granules, already noted along their length under ultraviolet light, fluoresce with green color, suggesting the presence of DNA, possibly deposited on inert substances. Exposure of alcohol-fixed smears to DNAse removes most of this fluorescence, supporting belief in the presence of DNA in the granules. As the long forms age with further incubation, and degenerate, the proportion of bacilli fluorescing with red color (nonspecifically?) increases.

The second approach to the problem of the metabolic processes during elongation of *M. lepraemurium* has been chemical. Technical difficulties included, first, the transfer to larger-scale production, on account of the requirements of a critical ratio of surface area of medium to weight of bacteria during elongation, and, second, the contribution of the liver tissue to some of the chemical analyses. The first difficulty was overcome sufficiently by using wide vessels with a large air space over a small volume of medium; the second difficulty was reduced substantially by using suspensions having more than 10^{11} bacilli per ml. after differential centrifugation (provided by R. J. W. Rees). Under these conditions, approximate doubling of length of bacilli, and of their dry weight estimated electron-micrographically (by R. C. Valentine), were found in two weeks, as in small-scale experiments.

An attempt was made therefore (in collaboration with J. Mandelstam) to determine which macromolecules were being synthesized during elongation and whether any increase found would account for the doubling of cell mass. Total protein, total nucleic acid, and muramic acid (the latter by A. J. Garrett), were examined. None of these showed any increase, indicating that cell constituents other than protein, DNA, RNA or mucopeptide are probably responsible. The constituent(s) could be polysaccharide or polyphosphate (the latter would account for the granules, which, as suggested above, might be carrying DNA), or could be small molecules such as phospholipids, amino acids, salts, etc.

The fact remains that there is doubling of the bacillary length, and a change in the cell wall must be occurring. Stretching is a possibility (though electron microscopy does not suggest any thinning); or a breakdown of cell wall material may occur, with resynthesis of products to become redistributed over twice the area. These considerations are of interest to the bacterial physiologist, but not necessarily to the *in vitro* cultivation of *M. leprae*.

Our present conclusion is that the conditions that give this probably very low-grade form of living growth *in vitro* may perhaps merit further attention as providing a clue to those which would ultimately give multiplication. On the other hand, they may be substantially irrelevant. Perhaps the results of other work to be given in the ensuing discussion will clarify the situation.

REFERENCES

1. Anderson, E. S., Armstrong, J. A. and Niven, J. S. F. Fluorescence microscopy: observation of virus growth with aminoacridines. 9th Symposium Soc. gen. Microbiol. (London) (1959) 224-255. Cambridge University Press.
2. Chang, Y. T. The mouse macrophage as host cell for *Mycobacterium lepraemurium*. Trans. Leonard Wood Memorial - Johns Hopkins University Symposium on Research in Leprosy, Baltimore 1961, 118-125.
3. Hart, P. D'Arcy and Valentine, R. C. Growth (without multiplication) of *Mycobacterium lepraemurium* in cell-free medium. J. Gen. Microbiol. **32** (1963) 43-53.
4. Young, M. R. and Smith, A. U. The use of Euchrysine in staining cells and tissues for fluorescence microscopy. J. Roy Microscop. Soc. **82** (1963) 233-244.

DISCUSSION

Dr. Weiser. Thank you, Dr. Hart. It was a very interesting paper. The discussion will now be opened by Drs. Laszlo Kato and G. Richard F. Hilson. Dr. Kato is Head of the Department of Physiopathology of the Institute of Microbiology and Hygiene at Montreal University. Dr. Hilson is temporarily with the Virology Section of

the Communicable Disease Center of the United States Public Health Service in Atlanta, Georgia.

Dr. Kato. The observations presented by Dr. D'Arcy Hart are most valuable for all of us. I do not feel competent to discuss the details of this problem, but will take the liberty of presenting some of our observations that seem pertinent.