The Effect of Various Additives on the Morphology of *M. lepraemurium* in the Medium of Hart and Valentine

G. R. F. Hilson, M.D.

The medium described by Hart and Valentine ([6]) appears to provide the best cell-free environment so far devised for *M. lepraemurium* to produce some degree of growth. Failure of multiplication to occur could be due to the presence of inhibitors, or the absence of essential nutrients. Having first confirmed that I could obtain the elongation effect with this medium, I carried out an experiment in which various additives were included with it, to see what influence they would have on the lengthening and degeneration of the bacilli, and with the very faint hope that one or other of the added materials might actually permit multiplication.

The medium and experimental methods were essentially as described by Hart and Valentine ([6]). The various substances under test were added in 2 ml. volumes to 6 ml. amounts of medium in half ounce screw-cap bottles, controls receiving 2 ml. distilled water instead. The final pH of test and control "cultures" varied between 6.05 and 6.40. A suspension of *M. lepraemurium* grown for 6 months in mouse testis was added to give a final concentration in each bottle of 4.6×10^7 bacilli per ml. The testis was homogenized in Hanks BSS + 1.5% (w/v) bovine albumin, and the homogenate freed of clumps and gross tissue fragments by slow centrifugation. On a rough count the supernatant contained about 10^{11} AFB/ml. The bacilli were washed once by centrifugation and resuspension in BSS-albumin. After an accurate count they were further diluted so that the addition of O.2 ml. to each "culture" pro-

duced the desired bacillary concentration. Test and controls were set up in duplicate, and all bottles were incubated at 37°C. Samples were taken at the intervals shown in Table 1, up to 16 weeks. Using Ziehl-Neelsen preparations (carbol-fuchsin applied for 30 minutes at room temperature), the total bacillary count and average bacillary length ([8]), and proportion of solidly-staining bacilli ([9]) were determined for each sample. A few parallel estimates by electron microscopy of length and of the proportion of degenerate forms were also made. Additives (Table 1): (a) "Formalin": formaldehyde to a final concentration of 2% (w/v); (b) "Adenine/purine": a mixture of adenine, two other purines, and three pyrimidines used in the development of a defined medium for the cultivation of *Haemophilus influenzae* by Butler ([2]). It was used (i) diluted so as to give a concentration similar to that optimal for *H. influenzae* in the defined medium and (ii) at a concentration 3 times as great (indicated by "3x" in the table); (c) "Spleen" and "liver": the spleens and livers of anesthetized rats were removed, chopped up finely at once with a sharp knife and rinsed several times with chilled Hanks' BSS until no more blood could be seen in the rinsings. One volume of washed organ mince was then homogenized with one volume of distilled water in a nylon grinder ([7]). The homogenate was centrifuged at 1,500 G for 20 minutes and the supernatant used for testing; (d) "Human serum": pooled human serum heated at 56°/30 min.; (e) "Yeast extract": fresh yeast extract prepared as described in ([1]). Dried yeast extract (Difco) was used as a 10% (w/v) solution in distilled water. Rationale: The purine/pyrimidine mixture was used to correct a hypothetic deficiency leading to a disordered nucleic acid metabolism and consequent failure of division; the tissue homogenates, relatively free of possible serum inhibitors ([3,4]), might provide some degree of imitation of *in vivo* nutrient conditions; yeast extracts were used because of their stabilizing effect on *M. lepraemurium* found by Hanks ([4]); and human serum was used to see whether its reported inhibition of the hydrogen transfer capacity of rat leprosy bacilli ([3]) was paralleled by an inhibition of lengthening in this system, and for future comparison with rat serum.

The results are summarized in Table 1. The values shown were obtained on samples taken from alternate bottles during the first 4 weeks, and then on pooled samples from both bottles thereafter. No significant change in total numbers of acid-fast bacilli was found in any bottle. The readings on the formolized control show that the initial average length of the bacilli was 1.6μ, and indicate the amount of variation to be expected in serial estimates of the proportion of nonsolid organisms. The positive controls (unmodified medium) show the expected lengthening to about twice the initial value; the rate of conversion to nonsolid bacilli is similar to that described by Hart and Valentine ([6]) if the proportion of about 10 per cent initially nonsolid forms is subtracted from the values. Bacillary elongation was inhibited almost completely by adenine/purine, spleen and liver; partially by human serum and fresh yeast extract; and not significantly by dried yeast extract. In the four sets showing lengthening, the maximum length was reached by the second week, except in the case of the dried yeast extract, where it was delayed until the 12th week. The most interesting finding was that, with the exception of the adenine/purine sets, the additives which suppressed lengthening most also preserved the solidly-staining state of the bacilli best. That this was not a purely technical effect exerted on the staining process was shown by the electron microscopy of some of the samples: the proportion of degenerate bacilli detected by this means correlated with the proportion of organisms showing the nonsolid staining reaction. This positive correlation between inhibition of growth and delay of degeneration is the opposite of the findings of Hart and Valentine ([5,6]) when they investigated the variables involved in achieving maximum elongation. This does not necessarily imply conflict, however, since the variables considered here are different. Hart and Valentine themselves pointed out that although the lengthening phenomenon was to some extent a vital process, it did not necessarily

TABLE 1. *The effect of various additives on the morphology of* M. lepraemurium *in D'Arcy Hart medium.*

Additive	Week							Maximum length (μ)
	1	2	3	4	7	12	16	
Formalin (control)	8[a]	—	—	14	13	10	11	1.6
Adenine/ purine 3x	—	—	—	27	81	86	—	1.8
Adenine/ purine	20	36	50	42	75	78	88	1.8
Spleen	11	11	24	39	45	53	60	1.8
Liver	10	24	37	42	62	60	60	1.8
Human serum	15	23	42	45	79	66	82	2.3
Fresh yeast ext.	40	39	—	50	75	65	91	2.3
Dried yeast ext.	12	27	52	52	81	82	92	2.8
Nil (pos. control)	23	34	53	63	84	86	86	3.0

[a]The numbers in the body of the table indicate the per cent nonsolid organisms found at the times indicated. Incubation at 37°C; no significant change in total numbers of acid-fast bacilli in any series.

take one on the road to bacillary multiplication, but might be a dead end. It is not clear whether prolonged preservation of vitality is incubated mixtures, such as seems to be implied by these results, would be any surer guide to our goal.

The purine/adenine and yeast extract additives seem to have been unhelpful or suppressive, without any saving effect on the rate of degeneration. The two extracts differed considerably in their influence on bacillary degeneration but this need not be considered too surprising, since their mode of manufacture differs. The fresh extract is obtained by boiling, and the dried is an autolyzate. It may be significant that the latter, which was merely inactive in this system, was the type of supplement found to have a stabilizing action by Hanks (4). A more pronounced suppressive and lethal action might have been expected of the human serum additive, which constituted 20 per cent of the medium (3): however, it is conceivable that serum inhibitors may have little or no action at the lower pH values used in this study, or existing intracellularly. It was thought important to prepare the rat liver and spleen homogenates in such a way as to reduce the extent of blood contamination; it cannot be said from these results whether these precautions were important or not. However, both materials had a favorable effect in preserving solidly-staining bacilli. The spleen homogenate was the more active, and this may be significant: both organs are heavily involved in animals with generalized rat leprosy, but the spleen is the richer source of macrophages.

The results reported were obtained from a single experiment, as yet unconfirmed, and must therefore be regarded as tentative. My main object in presenting them is to provide some basis for suggesting that our discussion might include consideration of the roles of morphologic change or survival rates as interim guides towards the development of a true growth medium for *M. lepraemurium* or *M. leprae.*

In other words, to what extent, in making

changes in media or hopeful improvements, will it be valuable to look for preservation or survival: will it, in fact be any guide? That is a general question on which I wish to finish. I have one specific question that I would like to put to Dr. D'Arcy Hart. There was some difficulty, when lengthening had occurred, in assessing the non-solidity of organisms. It looked as if some of the organisms were barred in a systematic way, rather than beaded in the degenerate way, and I wonder if he has any views on this, i.e., whether or not this corresponded in fact with the kind of granules that he described.

Acknowledgment. This work was carried out with the aid of a grant from the Endowment Fund of St. Goerge's Hospital, London, S.W. 1, England. My thanks are due to Miss Irene Mazurkewich for her technical assistance.

REFERENCES

1. Alexander, H. E. *In* Bacterial and Mycotic Infections of Man. 3rd ed. Ed. by R. J. Dubos. Philadelphia and Montreal, Lippincott, 1958. p. 480.
2. Butler, L. O. A defined medium for *Haemophilus influenzae* and *Haemophilus parainfluenzae*. J. Gen. Microbiol. **27** (1962) 51-60.
3. Hanks, J. H. Influence of physical and chemical factors on the hydrogen transfer capacity of murine leprosy bacilli. Internat. J. Leprosy **22** (1954) 162-173.
4. Hanks, J. H. Relationship between the metabolic capacity and the infectiousness of *M. leprae murium;* refrigeration studies. Internat. J. Leprosy **22** (1954) 450-460.
5. Hart, P. D'Arcy and Valentine, R. C. Elongation of a leprosy bacillus (*Mycobacterium lepraemurium*) in a cell-free medium. Nature (London) **185** (1960) 58-60.
6. 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.
7. Hilson, G. R. F. and Elek, S. D. A nylon tissue grinder. J. Gen. Microbiol. **11** (1954) 247-249.
8. Hilson, G. R. F. and Elek, S. D. Intratesticular multiplication of *Mycobacterium leprae murium* in normal and suramin-treated animals. Internat. J. Leprosy **25** (1957) 380-391.
9. Rees, R. J. W. and Valentine, R. C. A modified Ziehl-Neelsen staining method for identifying dead *Mycobacterium leprae murium*. Internat. J. Leprosy **30** (1962) 414-417.

DISCUSSION

Dr. Weiser. Thank you, Dr. Hilson. The meeting is now open for general discussion.

Dr. Dharmendra. Dr. Hart's paper was a very beautiful one. I am not in a position to comment on the findings reported by Dr. Hart, but there is one particular aspect relating not only to this paper, but to all of the work and all of the papers that report work on the so-called rat leprosy. I wish to put it to this conference to give considered thought to the suitability and desirability of the terms *M. lepraemurium* and the so-called rat leprosy.

There is no justification for calling the acid-fast bacillus causing lesions in rats, the rat leprosy bacillus, or the disease itself rat leprosy. The advantages of working with this organism are very doubtful. The only elements common to the leprosy bacillus and the so-called rat leprosy bacillus are that both are acid-fast and both have not been cultivated in the culture media used commonly for growing acid-fast bacilli in general, or in any other special media.

There is no similarity between the lesions caused by the leprosy bacillus and those caused by the so-called rat leprosy bacillus, unless the position is taken that any uncultivable acid-fast bacillus producing lesions in any animal, and the disease so caused, will be called the leprosy bacillus and leprosy, respectively, qualified by the addition of the name of the particular animal. There is no justification for the terms "*Mycobacterium lepraemurium*" and "rat leprosy." There was no doubt a tendency toward that position when a particular disease found in buffaloes was termed as "lepra bubalorum," but that tendency is fortunately no longer evident.

Moreover, studies made with so-called *M. lepraemurium* have not in any way contributed to our understanding of the leprosy bacillus or the disease produced by it. They have not provided a satisfactory method even for screening drugs for the treatment of leprosy. With Shepard's foot pad method for screening antileprosy drugs, screening with the so-called rat leprosy bacillus becomes superfluous.

On the other hand, the terminology used for the causative organism and the disease produced by it in rats, is confusing to persons not very conversant with the subject. The general impression created will be that the rat leprosy bacillus is a variant of the leprosy bacillus, just as bovine and avian tubercle bacilli are variants of the human type tubercle bacillus. There is no evidence at all justifying such a view. Moreover, the existence of the so-called rat leprosy bacillus strain in a laboratory dealing with animals inoculated with *M. leprae* creates difficulty. It is likely to introduce errors. I am of the view, therefore, that there is need to give serious thought to the matter of changing the terminology of the particular disease found in rats and the causative organism thereof. The present situation leads to unnecessary confusion, without offering any advantage. While making this suggestion, I am conscious of the fact that this terminology originated in India, and that a considerable amount of work was done and reported from the Calcutta School of Tropical Medicine. All the same, I cannot help feeling that the position is not tenable, and for the audience here who may not be familiar with the origin of the terms "rat leprosy" and "rat leprosy bacillus," I would like to say a few words on the subject.

During investigations on plague epidemics, which used to be very common in India, the rat population in Bombay (as in some other cities) was examined on a large scale. In the course of these investigations, acid-fast bacilli were found in some of the rats examined. These bacilli could not be cultivated in any of the media commonly used in the cultivation of the tubercle and other acid-fast bacilli. In this respect, the organism resembled the leprosy bacillus, and hence the terminology. It was believed that the organisms were closely related. However, evidence to this effect is misleading. As I have explained, this terminology is not correct, has no advantage, and therefore needs to be changed.

Dr. Weiser. I shall ask Dr. Hart if he will comment on Dr. Dharmendra's discussion.

Dr. Hart. I shall make only one comment, viz., to try to answer the question whether studies on rat leprosy really have any bearing on infection with *M. leprae*. My view is that in the "circling" motions we go through in our observations with *M. johnei*, its growth factors may be relevant to the problem of growing *M. leprae* in the future. I would certainly think, too, that we cannot be too exclusive in our work with other mycobacteria.

Dr. Binford. In the leprosy research conferences at Carville in 1956 and 1958 the subject raised by Dr. Dharmendra was discussed, i.e., why leprosy investigators worked on rat leprosy. At that time I commented that a great amount of the research work in cancer that has been carried on all over the world and in the United States, has been largely in experimental mouse cancer. If it is rational to use mouse cancer for experimental work in neoplasm, as appears well accepted in most scientific circles, I think the study of rat leprosy, if I may use that term, is justified.

Dr. Goldman. I would like to ask Dr. Hart a specific question on which I think I can get a yes or a no answer and then have a more detailed one later. Dr. Hart, can these elongated bacilli be fragmented mechanically? Particularly the ones that are elongated during growth at pH 7 but not at pH 6. Has their respiration been studied so that you know if they are respiring or if they are in a different metabolic state? Changes in intracellular granules due to changes in respiratory levels are fairly frequent, as I am sure you are aware. I have another question, but I need the answer to these two first.

Dr. Hart. I doubt if I can give you very much satisfaction. As to fragmentation, we tried sonic vibration but no granules have been isolated, but we shall try again. I might add that there has been no sign of any cross walls. I am not sure that I quite understand your second question. At pH 7 there was little or no elongation of the bacilli. Even at pH 6 there are some that do not elongate, but those are the ones that are usually dead from the start.

Dr. Goldman. I take it that the 7.5 per cent sucrose in the medium is essential. The statement was that it is more uniform, but not essential. It seems to me, from listening to you, that you were doing these experiments in an 0.2 *M* sucrose medium, and that you were getting a generalized elongation that showed no change in protein and nucleic acid. There is no increase in mass while you are getting a change in permeability. All of this indicates something. I am not sure if I want to call it protoplast formation, but it certainly tends toward that. What you are doing seems to be getting a change in cell volume without a change in mass; I am not even sure if you are getting it in volume. You may not have the dimensions firmly enough in hand so that you can tell its volume, but I wonder if, in your treatment in this medium, you are moving toward a degeneration of the outer wall of the cell, retaining the membrane and obtaining a sort of protoplast formation that is much moore freely permeable and stabilized in your 7.5 per cent sucrose medium. And if this is so, or if there is a chance that it is so, then direct electron microscopy of the wall and the membrane should give you some indication of possible outer morphologic changes.

Dr. Hart. I think that is an attractive possibility. We thought of this, but I do not think the facts really support it. First, our medium is a hypertonic solution, but elongation can take place on the same medium (though without sucrose), solidified with agar. But I think the crux of the question is that we believe there *is* an increase in cell mass as well as in volume. This depends on how much reliance you put on the electron microscopic estimates of dry weights. Dr. Valentine also sees no evidence that the cell wall has thinned, or changed or behaved in any other way that could be in common with protoplasts. So we abandoned that hypothesis, although we did think of it.

Dr. Middlebrook. I would like to ask Dr. Hart if enough material is absorbed by the cells from the host tissue, i.e., the cells you use as your control to explain the failure of any increase in total protein or carbohydrate after they have grown. Is it possible that enough material is absorbed by the original cell, so that the total growth could have been at the expense of material absorbed to the cells, in which case you would observe no increase in the total protein? This is really more of a technical matter, and I would like to know a little more about the actual technic of measuring the total amount of protein of the original material, and in measuring it after elongation has taken place.

Dr. Hart. Organisms were collected and washed once with phosphate buffer and then put at −20°C until the end of the experiment, when they were taken out. We have assumed (though it is difficult to prove formally) that after much treatment exogenous protein, etc., does not remain adsorbed to the cell surface.

Dr. Middlebrook. I have had some experience with Dr. Segal's method of preparing living *M. tuberculosis* from mouse lungs in a suspension that looked as if it were pretty free from host material, as far as we could tell by staining the material with acid-fast staining technics and looking at the bacterial cells with ordinary light microscopy. I was not impressed by the amount of material on the cells, but when we made lipid extracts of those cells, we found a lot of lipid, including much cholesterol, which I am sure the cells did not synthesize. I think one can be misled into thinking there is not much host material on the cells, when in reality much is adsorbed to the cells that was obtained from tissues.

Dr. Hart. Can you think of any way to get rid of it?

Dr. Middlebrook. I could not think of any way of getting rid of it.

Dr. Hart. What you have in mind, I suppose, is that the contaminating tissue material may have been autolyzed during incubation to an extent that would offset a real synthesis of bacterial protoplasm.

Dr. Middlebrook. You would have the same total amount of protein, when actually there had been an increase in the actual amount of the cells themselves.

Dr. Hart. It seems unlikely that an exact balance of this sort should have occurred, to account for our findings, though it is difficult to rule this out. However, with muramic acid, in which we also found no increase, we are on much firmer ground, since this is not a constituent of mammalian tissue.

Dr. Weiser. I must close the discussion at this point. Our next speaker is Dr. Claude V. Reich, Director of the Leonard Wood Memorial-Eversley Childs Sanitarium Research Laboratory at Cebu City, Cebu, Philippines. He will speak on "Approaches to cultivation of *M. leprae* in a new laboratory."