

Stability of *Mycobacterium leprae* and Temperature Optimum for Growth

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Two general areas will be discussed that need to be considered in attempts to cultivate *Mycobacterium leprae*: first, the stability of *M. leprae* at different temperatures, and second the temperature optimum of its growth.

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The stability of the organism is a matter of special concern in leprosy research. Many of the research laboratories are located far from the sources of supply in the important leprosy endemic areas, so that clinical material must be shipped long distances at considerable trouble.

In the results to be described, viability was tested by the ability of *M. leprae* to multiply in mice. Such multiplication is,

of course, indisputable evidence that the organism was living. The experimental set-up for this purpose, however, must be one that makes perfectly clear whether or not multiplication has occurred, and an accurate and reliable method for counting AFB (acid-fast bacteria) is essential. The determination of solid ratios is also of great importance. Our evidence, obtained directly by the inoculation of mice (⁹), supports the view of Rees and co-workers (⁷) that only solidly staining *M. leprae* are viable. The determination of solid ratios takes only 15 minutes or so if the total count is high, so that the most favorable material for an experiment may be selected the same day it is available. It needs to be emphasized that *M. leprae* from different untreated patients, or from different groups of mice, may vary a great deal in their viability, at least a thousand-fold. Fortunately one can select the favorable materials on the basis of solid ratios and be assured of having material of high viability, under ordinary circumstances.

How these technics can be applied is shown in the following experiment (¹⁰): *M. leprae* in mouse passage material was incubated at 31°C in a medium based on SPG, a solution that contains sucrose, potassium ion, and glutamate and is effective in maintaining the viability of typhus rickettsiae. Portions of the same material were also incubated in another medium based on 7H9, one of the series of mycobacterial media developed by Middlebrook and Schaeffer. The starting material contained 1.7×10^5 AFB/ml., and the solid ratio was 38 per cent. After incubation for 7 and 14 days, samples were taken for determination of the number of AFB/ml. and the solid ratios. It was found that the number of AFB had not changed much, although the solid ratios had probably decreased somewhat. The material counted at 14 days was also inoculated into mice, and viable *M. leprae* shown to be present, in numbers that were predicted by the two microscopic determinations.

The experiment showed that the viability of extracellular *M. leprae* can be maintained fairly well for 14 days at incubation temperatures, a result that might not have

been predicted from theoretic considerations extrapolated from findings with other mycobacteria to this so-called "obligatory intracellular" bacterium.

In tissue suspensions at 0°C the viability of *M. leprae* is maintained for 2 to 3 weeks without serious change (¹⁰). This has been shown with mouse passage material and with clinical material. In this period of time the solid ratio drops somewhat, apparently more slowly than the viability as tested by mouse inoculation. This stability at 0°C allows enough time for the shipment of materials from any part of the world that is not more than a few days from international airports. It facilitates work with mouse passage materials also. In our laboratory we maintain about 30 strains of *M. leprae* in continuous passage, and this amounts to about 5 passages a month. From these passages and from other experiments we may have a dozen harvests to be carried out each month. The harvests are carried out over the course of a few days, and the materials saved at 0°C until the microscope results are available, at which time the materials are selected on the basis of AFB counts and solid ratios.

A number of freezing experiments have been carried out (¹⁰) to see if the freezing methods used to preserve other microorganisms could be used for *M. leprae*. The results were at first discouraging. The methods used at that time were those used to preserve viruses and rickettsiae, i.e., fast-freezing and storage at -60°C (in a mechanical chill-chest); several cultivable mycobacterial species were found to survive very well under this treatment. *M. leprae* survived sometimes, but only in such low numbers as not to be useful. We then turned to methods used for the preservation of animal cells, i.e., slow-freezing in the presence of glycerol or dimethyl sulfoxide. The particular adaptations had been employed successfully in our laboratory for preservation of tissue culture cells. Out of this came the finding that the addition of glycerol was distinctly beneficial, but the rate of freezing was not important. In recent confirming experiments, the viability has been maintained with no de-

tectable change for two months at -60°C . Our general experience in routine work has been that the viability of materials kept in the -60°C box has not been maintained for long periods even in the presence of glycerol, although direct experiments on this point are not yet completed. This would indicate that there is slow loss of viability in the -60°C box, and as a result we have recently turned to liquid nitrogen storage, that is, -193°C . Results with liquid nitrogen storage are not yet available, but physical considerations lead one to think that at this temperature the results will be much better than those with the -60°C box, since the activity of all molecules is very much less at -193°C .

Next I would like to describe some investigations of the temperature optimum of *M. leprae*. The possibility that *M. leprae* might have an optimum lower than 37°C is a rather obvious idea, which has suggested itself to many people over the decades. Probably the best clinical accounts are those of Paul W. Brand⁽³⁾, and he describes how the particular type of tissue involved in leprosy is its coolest part. The tissues involved by leprosy are the skin exclusive of the flexion creases, the superficial but not the deep courses of the peripheral nerves, and the upper but not the lower respiratory passages. Of course, the spleen and liver are involved in bacteremic states, and many visceral organs are attacked in amyloidosis, but neither of these conditions depends upon local multiplication of *M. leprae*. The temperature descriptions that I was able to find in the literature were not satisfactory because they referred to normal persons under carefully controlled environmental rooms, or to leprosy patients in sanatoria. In general the temperature sensing element did not provide sufficient spatial resolution, or when it did the sites were not chosen according to predilection for leprosy involvement, and frequently the sensing element was attached to the skin in a way that would disturb the local temperature by preventing local cooling, especially of the evaporative type. It seemed more instructive to carry out measurements in endemic areas so that the contributions of such fac-

tors as air temperature, diet, physical activity, to epidemiologically active domestic environments would automatically be taken into account. The temperatures were measured with the thermistors of the Yellow Springs Instrument Company². The probe used was in the form of a 22 gauge needle, in which the sensing element is located in the bevel. The bevel was placed on the skin, and usually moved slowly across the surface until the meter steadied. Although the surface of the skin is usually cooler than underlying tissue, the increase in temperature in the first 5 mm. of dermis or subcutaneous fat averages only about 1 degree C at environmental temperatures similar to those encountered here⁽¹⁾. With this probe there is adequate spatial resolution and a short time lag; these factors make it preferable to their so-called skin probe. The more elaborate and expensive devices, such as the thermographs, require a main supply, and are therefore not usable in many endemic regions.

Two geographic areas were studied: one at Karimui in the New Guinea highlands, through the courtesy of Dr. D. A. Russell and his staff; the other at Talisay near Cebu in the Philippines, through the courtesy of Dr. R. S. Guinto and his staff. Air temperatures at these latitudes are relatively constant throughout the year. All temperatures were recorded in the daytime; the air temperatures recorded were somewhat less than normal for the daytime in the Philippines, but above that for night.

The two areas had been unusually well studied epidemiologically, and it was possible to concentrate on infected families. Of the 19 subjects 9 had leprosy (4 tuberculoid and 5 lepromatous); 3 had received some treatment. Two newly developed, previously unreported cases were discovered in the course of the temperature studies. Twelve of the subjects were males and their ages ranged from 2 to 47 years and averaged 21. The first subjects studied in New Guinea were poorly nourished; so

²Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U. S. Department of Health, Education, and Welfare.

4 well-nourished young native men, not born at Karimui, were also studied there. All other subjects had passed their lives in the areas studied.

It was found that the presence of leprosy, state of nutrition, age, and sex had no important effect on the skin temperatures. Both at Karimui and Talisay the skin temperature of the observer was also recorded at selected sites and found to be about the same as that of the subjects, but these results are not included in the final tabulations.

The most important variables were skin location and air temperature (Fig. 1). There was a tendency for extremities to be cooler distally. When the air was warmer the extremities and nose and ears were warmer, as they were when there had been recent physical activity. The trunk and head (exclusive of nose and ears) were relatively constant at different air temperatures. There was not much sweating, but, when there was and it was exposed to the breeze, the temperatures were a degree or two lower.

The nose and ear, which are commonly heavily involved in lepromatous leprosy, averaged 29° to 33°C, and 25° to 31°C, respectively. The buttocks, knees, and elbows are also frequently involved in leprosy and these locations averaged 28° to 33°C. It was interesting that these extensor sites were usually several degrees cooler than the skin proximally or distally, or toward the corresponding flexor surface; e.g., the buttocks were cooler than the lower back, the thighs, or abdomen. Other commonly involved locations and their usual temperatures are: face, 32° to 34°C; hands, 31° to 33°C; and feet, 28° to 30°C. Three subjects had single, small tuberculoid patches, which ranged from 30.5° to 34.5°C.

Measurements were also made of sites that escape leprosy involvement. The area behind the ear was usually 35° to 36°C. The flexion creases also escape. Field measurements on the fossae opposite the elbow and knee were usually made on standing subjects with extended knees and elbows. However, the attitude of these joints during much of the 24 hours is one

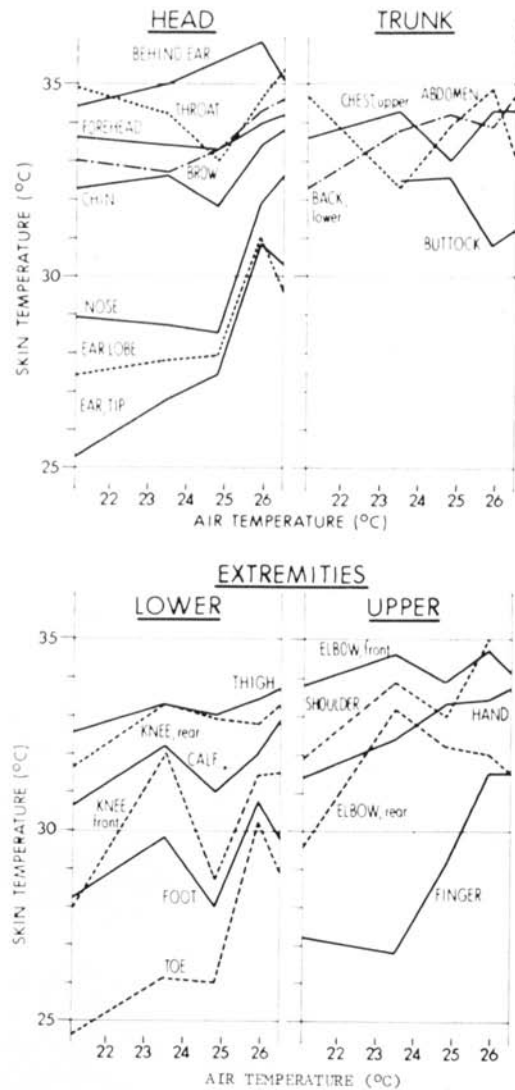


Fig. 1. Skin temperatures of persons in leprosy endemic areas. The results for each site are averaged for each air temperature. Those at air temperatures of 21.1°, 23.5°, and 24.8°C were taken in the New Guinea highlands; those at 25.9° and 26.5°C in the Philippines (Cebu). See text for further data on flexion creases. "Behind ear" refers to a site overlying the temporal bone, "forehead" to the middle lower region, "brow" to the skin of the medial portion, "nose" to the tip, "thigh" and "calf" to their lateral aspects, "hand" and "foot" to the dorsum of each, "toe" and "finger" to the 5th digit. Because they averaged about the same, only one of the following pairs is presented: medial and lateral brow, chin and medial cheek, upper and lower back, index and little finger, and 1st and 5th toes.

of partial flexion with some apposition of flexion creases. Later measurements were made after elbows and knees had been partially flexed for at least 10 minutes and the apposed flexion creases found to be 35° to 36°C.

Some leprosy endemic areas have higher air temperature than those recorded here for New Guinea and the Philippines, and it is hoped eventually to learn what particular skin site temperatures are in these warmer endemic regions. However, it seems possible that the temperatures of frequently involved skin sites will not be much different there. Ramanathan (⁶) records mean skin temperatures for normal resting subjects in Calcutta at air temperatures ranging from 23° to 36.5°C., i.e., mostly just above those in Figure 1. In this range he found that with each degree C of increase in environmental temperature there was an increase of only 0.03 degree in rectal temperature, and only 0.1 degree in mean skin temperature. At environmental temperatures above roughly 33°C, mean skin temperatures tended not to increase with air temperature.

This relative constancy of mean skin temperature at higher air temperatures is what might be expected from the consideration of the chief thermoregulatory mechanisms of man. At air temperatures below roughly 31°C (depending on humidity, external radiation, metabolic activity, etc.) there is cooling of the extremities, which minimizes the temperature differential between extremities and environment so that heat loss is minimized. Stimulated by decreasing skin temperatures (²), there is also increasing metabolic rate. If the temperature of the blood reaching the base of the brain also falls, the metabolic response to low skin temperatures is much greater. On the other hand at air temperatures above roughly 31°C, sweating begins and increases rapidly with increases of a few hundredths of a degree in the temperatures of the blood reaching the base of the brain (²). Evaporation of sweat cools the skin, and vascular responses shift an increasing proportion of the cardiac output to pass through the skin. The net effect in both temperature ranges is a

stable core temperature. Whether in hot climates the more frequent operation of the evaporative type of temperature control results in minor differences in the pattern of distribution of leprosy lesions is not clear; major differences have apparently not been described.

The nasal mucosa was measured in Atlanta on normal subjects. This is a difficult measurement because the mucosa is a moist surface subject alternately to the inhaled air, which is relatively dry and cool, and to the exhaled air, which is warm and saturated with water vapor. The measuring device itself can prevent evaporation or obstruct air flow and thus disturb the temperature. The most satisfactory measurements were made with an infra-red thermometer (Barnes Engineering Company)². At an air temperature of 20°C the nasal mucosa ranged from 26.5° to 33°C in each respiration; at 30°C it ranged from 31.5° to 36°C. For more precise location the needle thermistor probe was inserted through a nasal speculum and touched on sites high on the cartilaginous portion of the septum and on the middle turbinate, sites frequently involved in lepromatous disease. The measurements agreed with those made with the infrared thermometer, except that the excursion during each breath was not so great.

Measurements made in mice. *M. leprae* also multiplies in the mouse foot pad, a cool tissue at ordinary air temperature. Modifications of the temperature of the pad tissue can be achieved by keeping the mice at different ambient temperatures (⁴), and we have determined the effect of such treatment on the growth of *M. leprae* (⁸).

Environmental temperatures for the mice at 4°, 20°, and 25°C were obtained in rooms held at these temperatures. Bacteriologic incubators were used mostly for 10°, 15°, 30°, and 35°C, the former two being placed in the 4°C room.

In the first experiment the amount of bacillary multiplication was greatest in the mice at 20°C. It was slightly less at 10°, 15°, and 25°C, definitely slower at 30°C, and not observable in mice at 35° and 4°C. Since most of the mice at 4°C in this experiment were lost by accident, a second

experiment was carried out. Growth of *M. leprae* in the mice at 4°C was not observed for 330 days, whereas growth in the controls was normal.

Since the passage strains had been maintained in mice at 20°C, it seemed possible that there had been selection for bacilli growing in mice at this temperature. Accordingly several experiments were carried out with primary clinical material as the inoculum. In these experiments the 10°C mice were kept in a room in which there was mechanical convection. Multiplication was again most rapid at 20°C, and it took about twice as long to develop at 10° and 30°C. The multiplication in mice at 10°C was slower than it had been in the entire experiment, probably because the greater air circulation cooled the cages more effectively. Thus, there was no evidence that the *M. leprae* in passage had been selected for growth in mice at 20°C.

Temperatures in mouse foot pad tissues. In previous work (⁴) we found the temperature of those tissues to be within a few degrees of ambient temperatures between 10° and 35°C. We have since observed mice more closely and have learned how to take them up without exciting them. At ambient temperatures of 25°C and lower, mice spend most of their time in huddles, where the rear foot pads and the bedding are usually warmed by the huddle. A new series of measurements was carried out, again with the thermistor probe in the form of a 22 gauge hypodermic needle. Only undisturbed mice were measured; those from huddles or sitting alone were taken up directly before they had a chance to walk on surrounding bedding. The mice were allowed to acclimate for several weeks at the stated temperatures, and the measurements were carried out in a room adjusted to the temperature.

In the mice at 10°C a wide range of temperatures was recorded, but the mice in huddles had foot pad temperatures averaging 24.6°C. In mice at 20°C the range of temperatures was much smaller, and those in huddles averaged 30.0°C. In mice at 30°C the pad temperatures averaged 36.0°C for mice sitting quietly.

The temperature results are summarized

in Table I. The results indicate that the upper limit to the optimal temperature range of *M. leprae* is at 35° to 36°C. This would account for the distribution of leprosy involvement in the patient. It would also account for the failure of *M. leprae* infections to spread from the foot in the mouse.

TABLE I. Summary of evidence on the temperature optimum of *Mycobacterium leprae*.

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- I. Growth of *M. leprae* in mouse foot pads:
 - A. Most rapid when air temperature is 20°C (average foot pad temperature of 30°C).
 - B. Slower when air temperatures are 10° or 30°C (average foot pad temperatures of 25° and 36°C, respectively).
 - C. No growth when air temperatures are 4° or 35°C.
 - II. Growth of *M. marinum (balnei)* (⁴):
 - A. In mice, temperature optimum very similar to that of *M. leprae*.
 - B. In vitro, temperature optimum is 25° to 35°C.
 - III. *M. leprae* in man:
 - A. Sites most heavily infected range from 25° to 33°C.
 - B. Sites that escape are 35° to 36°C.
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A word should be said about temperature optima of biologic systems. In general the rate of all biologic activities increases slowly with temperature through the optimal temperature range of the particular activity, and this is true with single enzymatic reaction with purified reactants or with overall biologic activities such as the rate of chirping of crickets (⁵). The increase in rate through the optimal temperature range is a function of the activation energies of the molecules involved, and for most biologic systems this is on the order of 5 to 20 kcal. per mole. Just above the upper limit of the optimal temperature range, however, there is a rapid decrease in the reaction, on the order of 50 to 150 kcal. per mole. Thus one would expect that the incubation of *M. leprae* a de-

gree or two above the upper limit of the range of its temperature optimum would depress the growth rate much more profoundly than incubation a degree or two below the limit.

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DISCUSSION

Dr. Rees. Thank you very much, Dr. Shepard. I see, rather embarrassingly, that I am due to open this discussion. I would like to begin with a few remarks, some of them perhaps a little challenging to Dr. Shepard, and then throw the meeting wide open. The most important thing to say at this stage, is that *M. leprae* multiplies in the foot pad which to some of us is quite satisfying, and also that it was only very careful quantitative work that revealed this multiplication. I would make the point here for those of you who are not in this field, that this is truly very much a microinfection; unless those of us working in the field had really developed pretty sensitive methods, we probably would not be noting multiplication. This careful quantitative work is based on the number of bacilli put in and the number recovered and unless you put in less than 10^6 bacilli you won't even get multiplication. It is, I think, highly satisfying that all this quan-

titative work is related to numbers of organisms, which I must stress in the light of earlier discussions this morning are acid-fast bacilli. We are particularly grateful to Dr. Shepard, who has been able to show with *M. leprae* what we were able to show with *M. lepraemurium* several years ago, and I would press the point that we would not have been able to study the phenomenon at all, if we had not used *M. lepraemurium* as a model. By its use at a very early stage, we were able to determine those organisms that were viable and those that were dead. Now this is a very important contribution, because we cannot grow the organism, and it is therefore rather fundamental to have a short cut for determining viability. I will not go over our work, but simply say that first on a morphologic basis with the electron microscope, and then by simple Ziehl-Neelsen staining, we were able to show a complete correlation equating viability and infectivi-

ty for the murine leprosy bacillus, with reference to those organisms that were solidly staining or irregularly staining. Dr. Shepard undoubtedly has been able to show that this phenomenon applies also to *M. leprae*. This is an important contribution to study of the cultivation of *M. leprae*, for it provides an early indication whether or not our culture procedures are in fact standing any chance at all. If it can be shown that the organisms die rapidly, then one has a quick sorting method for selecting suitable media.

There is a controversial subject that I would like to bring up as a challenge straight away. I agree absolutely with what Dr. Shepard has said about the site in which *M. leprae* seem to be found and to have multiplied, viz., the superficial sites. Undoubtedly, however, there are exceptions to this in man. It is particularly impressive, for example, that many of the nerves involved are not necessarily the most superficial. I am also much impressed by the observation of my clinical colleagues, of the so-called carpal tunnel syndrome, which one sees in other clinical conditions which particularly pick out the nerves that are involved in leprosy. Trauma, however, seems to be a much more important feature. So I think one must consider trauma as well as temperature. Again Dr. Shepard referred particularly to the fact that acid-fast bacilli are found in livers and spleens in man, as we would have all expected in view of the huge number of bacilli in other sites. That is, there is a bacteremia, in which bacilli are picked up by the reticulo-endothelial system. Nevertheless, in January this year in India, I was most impressed by the systematic study that Dr. Job, who is with us today, has been making on liver biopsies from patients with all types of leprosy. In the beautiful histologic sections that he showed me I was strongly impressed by the bacilli within the liver. To my mind they were definitely distributed in such a way as to indicate that they were truly multiplying *in situ*, and they were good solid bacilli. So I am sure *M. leprae* can grow at 37°C.

I said I would not take up much time,

and I will not because tomorrow I speak again and will then refer to the subject in detail. I would point out now, however, that what Dr. Shepard has said of the growth of *M. leprae* in the foot pads of mice has been confirmed by others. It has been shown to grow in the same kind of way in the foot pads of rats and hamsters, and the ears of hamsters and mice. Recently, in a systematic study on nerves, we found, much to our astonishment, particularly large collections, almost macrocolonies, of well staining acid-fast bacilli in striated muscles of the mouse foot pad, and, although we have not checked the temperatures of the striated muscle, we would again not expect this site to be particularly low in temperature. Even more, we were impressed by recent work where we deliberately inoculated *M. leprae* into the thigh muscles of mice and were able, in some of them, to recover organisms with the same kind of increases that have been found in the mouse foot pad. Here again we feel that in the thigh muscle, particularly perhaps of the mouse, the temperatures are not likely to be low. Therefore, I would suggest that *M. leprae*, at least, may have a high and wider range of temperature in which it can grow, rather than at 32°-34°C only. I will stop here and throw this paper open for general discussion.

Dr. Weiser. I wish to make one brief remark. I had much experience with low temperature work in bacterial storage some years ago. And I can assure Dr. Shepard that if you can store these organisms for one minute at the temperature of liquid nitrogen, you can store them for a good long time.

Dr. Kirchheimer. I would like to say something with regard to the generation time of *M. leprae*. I believe a case can be made for the fact that it grows more rapidly than we have thought it does. If I am not mistaken, the generation time Dr. Shepard showed was calculated by using a multiplication index of 2. What this means, in other words, is that 100 per cent of the bacteria multiply all the time. I think it has been amply shown that even with

more readily multiplying bacteria this is not the case, and that a reasonable consideration would be that 60 per cent of bacteria like *Escherichia coli*, for example, multiply all of the time. Now, if we should make the assumption that only 10 per cent of the leprosy bacilli multiply all of the time, we would arrive at a generation time of about three days. I recall a letter from Dr. Wade, referring to a patient with reactive leprosy. Accidentally he had made skin scrapings in this patient before the patient had his reaction and scarcely any bacteria were to be found. Immediately after the patient went into reaction, the skin scrapings were full of bacteria. So, in this case, Dr. Wade believed the generation time of the leprosy bacillus may actually have been hours instead of days. This is not enough evidence, of course, but I think one might consider it, although not from a quibbling standpoint, as showing about how fast the leprosy bacillus can grow. The important fact is that perhaps there are conditions in the course of leprosy where the bacteria multiply especially fast. And since we are interested in cultivating the leprosy bacillus, I wonder if there are not at that time, in the tissues of the particular person, substances present that deserve to be added to prospective culture media, and not only to artificial culture media. As Dr. Moulder mentioned quite rightly this morning, we should be interested also in tissue culture. Of course we are doing this and I think we might very well make additions to our cell cultures and see if they enhance the chance of growing the leprosy bacillus.

Dr. Shepard. First, I would like to point out, Dr. Rees, in response to your first question, that the work I described could not have been done unless we worked with *M. leprae*. As to the matter of temperature—we have measured the deep temperatures in the foot and found them not very much different from the subcutaneous temperatures. The measurements in mice were actually made by the insertion of a needle probe; it did not matter much whether the needle tip was in the muscle or in the subcutaneous tissue. The systemic tempera-

ture of mice as indicated by rectal temperatures, is around 37°C, but it is not infrequently a little lower than that. It would not be surprising to me if the temperature of the thigh were 36°C. or lower. You remember that in an environment at 30°C the average temperature of the foot pad was 36°C. We found slower growth at a tissue temperature of 36°C, not absence of growth. As to generation time, it is a large subject. I shall perhaps come back to it a little later in the week. I would just point out now that we use this estimate as a convenient monitoring of the course of events in the infected mouse. We do not claim that this measured value is the true generation time. If you follow the growth curve with repeated harvests through the logarithmic phase, you find generation times of 12 or 13 days, and sometimes we have had that right from the beginning of the experiment. In the human patient, you do not know how many organisms were in that particular piece of tissue before the observation started. It is not possible to take out the same piece of tissue twice. Of course an organism may have a somewhat different generation time *in vitro* than *in vivo*. For example, *M. tuberculosis* in mice, under ideal conditions, can grow with a generation time pretty close to one day. *In vitro* you can get it to grow under the best conditions with a generation time of about 8 or 9 hours. But, in general, there is not a dramatic difference between *in vivo* and *in vitro* conditions for the same organism, and growth rate is one of the more constant characteristics of a given microorganism.

Dr. Rees. I would just like to say, Dr. Shepard, that the time taken for these increases in the thigh muscle was the same as in the mouse foot pad.

Dr. Pattyn. I would like to ask Dr. Shepard if the temperature at the site of the skin lesions in leprosy patients is any different from that of normal controls or from the neighboring skin not affected by the disease.

Dr. Shepard. No. We measured temperatures from normal skin across lesions

to normal skin and did not find any important changes from the normal pattern. The normal pattern of temperatures needs to be taken into account in a particular location. For example, going down an arm toward the extremity, we find that the skin becomes cooler, and going from the elbow toward the flexor surface it becomes warmer. I am sure there are lesions where there are neurologic changes that lead to vascular changes and resultant changes in temperature. Most of the lesions we studied were minimal.

Dr. Goldman. I would like to make a remark about two aspects which I think might perhaps give Dr. Reich an answer to the question he revised yesterday, viz., how we are going to recognize *in vitro* cultivated *M. leprae*. First, if we assume that some kind of *in vitro* culture medium can be inoculated with a small number, i.e., less than 100 *M. leprae* from a human or mouse source, and growth occurs, then possibly there is no genetic change. This is a problem often raised in leprosy—whether or not there is some genetic change on transmission from man to animal. Second, if the organism grown even looks like, say a gram-positive sporulating bacillus, and if it is then put into mouse foot pads and gives the pattern of growth that Dr. Shepard has so often demonstrated, can we show whether or not it is *M. leprae*?

Dr. Hart. May I ask a question about *M. lepraemurium* temperatures, of either you or Dr. Shepard. From what Dr. Shepard said, we would start using 30°C for *in vitro* work. It is very difficult, of course, and very confusing, because the solid organisms last very much longer, and you really will have to do counts, which is probably quite right. What I want to know is this—if there is information about the optimum temperature for lepraemurium multiplication in tissue culture or foot pads or anything? Does either of you know? Am I anticipating tomorrow?

Dr. Rees. You could be. It will come up tomorrow. The answer is that 37°C seems to be the optimum in tissue culture.

Dr. Hanks. *M. lepraemurium* grows in all of the available sites in which you may inoculate a mouse or rat. However, cool tissues are optimal for crop production, e.g., the anterior chamber of the eye and particularly the testis. Both are influenced by environmental temperatures, but under most circumstances are probably in the range of + 34°C.

Dr. Rees. I think we will have to break off now. In continuing this session I will call on Dr. B. R. Chatterjee, who is Cytologist at the Johns Hopkins-Leonard Wood Memorial Leprosy Research Laboratory in Baltimore. Dr. Chatterjee will speak to us on "Growth habits of *M. leprae*; their implications."