# RELATIONSHIP BETWEEN THE METABOLIC CAPACITY AND THE INFECTIOUSNESS OF *M. LEPRAE MURIUM*; REFRIGERATION STUDIES

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Correlations between the infectiousness (7) and the hydrogen transfer capacity (HTC) of incubated suspensions of *M. leprae murium* have been considered previously (8). These studies brought to light the toxicity of serum and also the improvement and conservation of HTC and infectiousness by purified serum albumin. Gray (4) demonstrated an inverse relation between the endogenous respiratory activity exhibited by washed suspensions of bacilli and the degree to which the existing inhibition of metabolism (8) is relieved by albumin. He also discovered a consistent enhancement of respiration in the presence of yeast autolysate (4). These two materials produced the most significant beneficial effects observed in the present investigation.

Refrigeration provides an interesting circumstance under which to study the relationship between metabolic activity and infectiousness, particularly because the bacilli do not burn up their endogenous reserves of lipids and polysaccharides. The data obtained in this study permit: (*a*) further definition of the principles by which infectiousness may be predicted from metabolic data, (*b*) analysis of the essential dependence of infectiousness on metabolic activity rather than on biochemical content, (*c*) comparison of the relative sensitivity of rat inoculation and HTC determinations, and (*d*) practical suggestions for improving the preservation of infectiousness during storage of these microorganisms by refrigeration.

## METHODS

Methods for comparing the infectiousness of experimental aliquots of murine leprosy bacilli have been described (7). In the present work the data were obtained by inoculating four or six sites per Wiersing rat (9) in the improved rotating pattern, and by the use of 9 or 18 rats per group. Results are expressed as the average weight of leproma per site at autopsy, which permits numerical comparison of different degrees of infectiousness.

Methods for measuring the HTC of mycobacteria have also been described (5).[2] The principle on which the method is based and the general procedures have been summarized in THE JOURNAL (8, 10). Duplicate 0.1 ml aliquots of washed *M. leprae murium* corresponding to nephelometer No. 40 or No. 80 were employed for each

[1] With the assistance of Miss Tobey Backerman.

[2] The original paper failed to state that the evacuated anaerobic bottles are refilled with 600 mm. partial pressure of nitrogen. Otherwise, there is excessive distillation of water vapor from the tests to the walls of the anaerobic vessels.

HTC determination. The bacterial content of cruder "concentrates" of bacilli must be expressed as the amount of infected tissue from which they were derived. All suspensions were brought to 0.5 ml before the addition of 0.1 ml of a 1 per cent solution of tetrazolium violet (TzV). This provided 1,000 μgm. of acceptor at 0.7 per cent.[3] The suspensions are capable of transferring hydrogen for periods of more than 14 days ([5, 8]), but the amount of formazan (reduced TzV) extractable in acetone after an empirically-chosen period of four days of anaerobic incubation at pH 7.5 was taken as a measure of the HTC.

All reagents were known to be nontoxic and suitable for biological use. Glycerol 50 per cent by weight corresponds to the 40 per cent by volume used by earlier workers. Purified bovine albumin 35 per cent sterile solution, and Bacto (yeast) Supplement B (an autolysate) were obtained from the manufacturers.[4] Solutions to be incorporated in HTC determinations were adjusted to pH 7.5 by means of KOH.

In each experiment with tissue homogenates in glycerol and sucrose, infected testes were removed from 5 to 7 rats. Organs from the left side and those from the right side were placed in separate pools. One pool was homogenized in cold glycerol and the other in cold sucrose solution, to provide 40 per cent tissue homogenates in glycerol 5.4M (50% by weight) and in sucrose 0.9M (31%), respectively. The cold homogenates, in fluid depths of 2.5 inches in screw cap tubes, were refrigerated promptly at 4-6°C for periods of 12-14 weeks. At this time the respective homogenates were diluted four times, to 10 per cent tissue, and the bacilli were recovered as rigorously washed suspensions ([5]). The usual depths of density barriers were employed, but in narrower tubes than those used previously.

TABLE 1.—*Influence of pH, glycerol and albumin on the preservation of* M. leprae murium *during refrigeration in tissue homogenates for 12 weeks.*

| Refrigeration solution | pH | | HTC[a] | Infectiousness[b] |
|---|---|---|---|---|
| | Original | Final | | |
| Glycerol 50% | 6.5 | 6.0 | 63 | 0.00 |
| | 7.5 | 7.0 | 110 | 0.14 |
| Bovine albumin 17.5% | 6.5 | 6.2 | 120 (156) | 1.10 |
| | 7.5 | 7.2 | 172 (224) | 2.80 |

a Micrograms of TzV formazan produced by bacilli from 1.0 ml of 5 per cent tissue homogenate. The figures in parentheses indicate yields anticipated in the presence of 2,000 μgm. of acceptor; see Footnote 3.

b Average weight of lesions/16 sites, each site inoculated with 0.1 ml of 5 per cent tissue homogenate. Observation period seven months.

In the experiments with tissue homogenates in glycerol and albumin, the entire pool of infected testes was homogenized in cold water to make 40 per cent tissue. This homogenate was immediately divided among tubes containing an equal volume of double-strength glycerol or albumin solution. One-half of the tubes containing each reagent were left at the natural pH of the combined solutions and homogenate

[3] Reference has been made to the binding of TzV by albumin ([8]). Had the determinations been carried out in the presence of 2,000 μgm. of acceptor, HTC values of albumin-refrigerated bacilli and values in the presence of albumin would have been increased by about 30 per cent.

[4] Armour and Company, Chicago, Illinois, and the Difco Laboratories, Detroit, Michigan, respectively.

(approximately pH 6.5), while the others were brought to pH 7.5 and again corrected to that level at the end of the first week. The terminal pH values are shown in Table 1. Bacilli were recovered by diluting the homogenates 20 times with water to lower the concentration of storage agents and soluble tissue components, by centrifuging to concentrate the bacilli, and by resuspending in M/15 $PO_4$ buffer at pH 7.5 to standardize pH and electrolyte. HTC was determined on duplicate 0.5 ml aliquots of these suspensions at a concentration corresponding to 10 per cent tissue, while each of the inoculation sites in 18 rats received 0.1 ml corresponding to 5 per cent tissue.

In experiments involving refrigeration of previously washed bacilli, similar dilutions and reconcentrations were carried out before testing the endogenous HTC and the response to albumin and yeast supplement. In final experiments on infectiousness after only 24 hours, the refrigerated suspensions at nephelometer No. 80 concentration were simply diluted to nephelometer No. 40 for HTC determinations and to nephelometer No. 5 for rat inoculation.

## EXPERIMENTAL RESULTS

At body temperature *in vitro* the respiration (4), HTC (6, 8), and infectiousness (7) of *M. leprae murium* deteriorate rapidly. Under optimal conditions only 40 per cent of the initial HTC of active suspensions remains after incubation for 24 hours. During refrigeration HTC declines much more slowly. The most active of the washed suspensions retained 85 per cent of the initial HTC after refrigeration for one week in M/15 $PO_4$ buffer at pH 7.5 (8).

### REFRIGERATION OF INFECTED TISSUE HOMOGENATES

Since dense solutions of sucrose are employed routinely in the recovery of purified suspensions of *M. leprae murium*, the first experiments were designed to inquire whether homogenates could be refrigerated in sucrose solutions as satisfactorily as in the classical glycerol solutions. After refrigeration for 12 and 14 weeks (three experiments), bacilli recovered from sucrose-refrigerated homogenates by rigorous washing and tested in phosphate buffer produced from 1.7 to 3.5 times more formazan than bacilli recovered from glycerol. Similar differences were obtained when rigorously washed bacilli were refrigerated and tested in the same way (Table 2).

However, if bacilli which have been stored in these solutions are tested in the presence of albumin and yeast supplement, they are then found to possess equal HTC. Furthermore, if the pH was adjusted to 7.5 before storage, there was no measurable difference in the infectiousness of sucrose-refrigerated and glycerol-refrigerated bacilli. An acidity of pH 6.5 (the natural pH of homogenates) was unfavorable for preservation of HTC, as well as of infectiousness (9).

If homogenates are refrigerated to preserve bacilli for animal inoculation, one would ordinarily dilute the suspensions to the desired concentration and proceed directly with injections. The experiments to compare refrigeration in glycerol and in purified albumin at two different pH

levels, therefore, were conducted without rigorous washing of the bacilli.[5]

After 12 weeks of refrigeration both HTC and infectiousness were found to be preserved most successfully at pH 7.5 (Table 1). This is the pH most favorable for sustaining the *in vitro* metabolism of incubated suspensions of *M. leprae murium* (4, 8). At both pH 6.5 and pH 7.5 the endogenous HTC of bacilli refrigerated in albumin was nearly twice that of bacilli refrigerated in glycerol. The albumin-refrigerated bacilli, however, proved to be even more infectious than might be anticipated from the HTC values.

### REFRIGERATION OF WASHED BACILLI

In three experiments, bacilli recovered from fresh testicular tissue by rigorous washing were refrigerated at pH 7.0-7.5 in the solutions that have been mentioned, in yeast supplement, and in albumin together with yeast supplement. After four weeks the suspensions were diluted 15 times, reconcentrated, and aliquots were then resuspended in two solutions: (*a*) the usual phosphate buffer at pH 7.5, and (*b*) the same plus albumin and yeast supplement. The former permitted measurement of the existing

TABLE 2.—*The importance of determining residual HTC in the presence of materials affording the maximal response now obtainable* in vitro.

| Suspensions tested | HTC[a] tested in the presence of: | |
|---|---|---|
| | M/15 $PO_4$ | Albumin and yeast supplement |
| Original | 416 | ? |
| (After refrigeration for four weeks in:) | | |
| Phosphate buffer | 174 | 304 |
| Glycerol 50% | 56 | 310 |
| Sucrose 31% | 213 | 318 |
| Albumin 15% | 173 (220) | 345 |
| Yeast sup. 1:6 | 205 | 344 |
| Albumin and Yeast supplement | 201 (260) | 396 |

*a* Micrograms of TzV formazan/0.1 ml of nephelometer No. 80; average of three experiments. Figures in parentheses indicate yields anticipated in the presence of 2,000 μgm. of acceptor.

[5] It was known from unpublished data that there is no endogenous reduction of TzV by 0.5 ml of residue from 10 per cent normal tissue homogenates after refrigeration, dilution and reconcentration in the manner described. Although reconcentrated sediments from infected homogenates are admirably suited to the present purpose, they are not acceptable for biochemical work in which the response of the bacilli to substrates is to be studied. A control series of determinations in M/60 succinate demonstrated that the relatively stable succinic dehydrogenase of tissue had been preserved by albumin, but not by glycerol.

HTC, while the latter mixture provided the maximal response which these organisms have been found to exhibit *in vitro*. The results are shown in Table 2.

As judged by the response in the presence of albumin and yeast supplement, neither glycerol nor sucrose was superior to the regular phosphate buffer. Glycerol-refrigerated bacilli, as before, exhibited in phosphate buffer much lower HTC than sucrose-refrigerated bacilli but they were capable of equal response in the presence of albumin and yeast supplement. Under this condition they were comparable to sucrose-refrigerated bacilli, just as in earlier measurements of infectiousness.

The HTC of albumin-refrigerated bacilli[6] was not consistently superior to that of phosphate or of sucrose-refrigerated bacilli, but when tested in the presence of albumin and yeast supplement such bacilli were consistently superior. Bacilli refrigerated in yeast supplement, although possessing higher HTC in simpler buffer, fell in the same class when tested for maximal response. Bacilli which had been refrigerated in combined albumin and yeast supplement, whether tested in buffer or in this combination, possessed the highest HTC.

In summary, the maximal responses indicate that the metabolic capacity of these bacilli fell into three categories, depending upon refrigeration in: (*a*) simple solutions, (*b*) in albumin or in yeast supplement, or (*c*) in a combination of these two reagents.

The metabolic and infectious qualities of washed suspensions which had been refrigerated for only 24 hours in solutions of these three catagories and also in the presence of protamine sulfate (Lilly) are shown in Table 3. Bacilli refrigerated for this brief interval do not differ in

TABLE 3.—*Influence of protamine, albumin and yeast supplement during refrigeration for only 24 hours.*

| Solutions | Individual experiments | | | | Averaged data | |
|---|---|---|---|---|---|---|
| | HTC[a] | | Infectiousness | | HTC | Infectiousness[b] |
| | A | B | A | B | | |
| $PO_4$ M/90 | 78 | 68 | 1.48 | 1.77 | 73 | 1.62 |
| Protamine sulfate 0.3% | 73 | 85 | 1.72 | 1.45 | 79 | 1.58 |
| Protamine sulfate 0.03% | 75 | 89 | 1.46 | 1.26 | 82 | 1.36 |
| Bovine albumin 6% | 112 | 100 | 1.97 | 2.22 | 106 (138) | 2.10 |
| Yeast supplement 1:6 | 133 | 171 | 2.02 | 2.19 | 152 | 2.11 |
| Albumin and Yeast supplement | 190 | 226 | 2.68 | 2.61 | 208 | 2.65 |

*a* Micrograms TzV formazan/0.1 ml nephelometer No. 40.
*b* Average weight of lesions per nine sites in each experiment.

[6] As measured in the presence of 1,000 μgm. of TzV; see Footnote 3.

infectiousness as markedly as those refrigerated for 12 weeks. Lest it be thought that the differences in this respect are too small to be significant, the results of two experiments are given individually, then as an average. Gray (4) had observed that protamine stimulates the respiration of certain suspensions of *M. leprae murium*, but we had been unable to obtain appreciable enhancement of HTC with this reagent. In the case of bacilli refrigerated in buffer and in protamine, overlapping among the numerical values for both HTC and infectiousness occurred. It is again evident that the results, both for HTC and infectiousness, fall into the three catagories defined by refrigeration for four weeks and shown in Table 2.

## DISCUSSION

Results of the present study contribute further evidence of the close relationship between the HTC and the infectiousness of *M. leprae murium*. Experience now permits definition of certain working conditions appropriate for the collection of metabolic data having significance with respect to infectiousness.

In preliminary work, both HTC and respiratory activity should be measured to make sure that the bacilli are carrying on both of these metabolic functions. A simple modification of the HTC procedure to provide such determinations has been described (8). Whenever one function appears to be enhanced and the other to be depressed or unmodified, biological significance must be questioned until further study provides an explanation, or indicates which method should be employed. Anaerobiosis, for example, enhances HTC (8) but damages respiration (4). The dependence of infectiousness on respiratory activity has been discussed (10), and an explanation of the observations has been given (8). Protamine sulfate usually enhances respiratory activity (4), but is shown in this study not to increase HTC or infectiousness. Protamine, therefore, merely facilitates some phase of respiration without improving the continuous series of steps by which internal energy is utilized.

When, however, both measures of metabolism are modified in the same direction, metabolic data have been found to be highly significant. Native serum, for example, damages severely both HTC and respiration, infectiousness is sharply diminished. Serum albumin and yeast supplement, on the other hand, improve both HTC and respiration; both are shown in this study to enhance infectiousness.

Other principles reemphasized by the present work indicate that, if bacilli have been exposed to various reagents, their activity should be measured after satisfying two conditions. The first is dilution, centrifugation, and resuspension to standardize the chemical background. The second is the testing of metabolic capacity in the presence of materials which induce the maximal response which the organisms can exhibit *in vitro*. These conditions are consistent with those demonstrated in

previous studies on the relationship between the HTC and viability of *M. phlei* (6).

In the present experiments the HTC of albumin-refrigerated bacilli was superior to that in simple solutions after both 24 hours and three months of refrigeration at pH 7.5, but failed to predict the remarkable infectiousness of those held in albumin for three months. Bacilli stored in albumin only 24 hours produced 74 per cent greater lesion weights than the controls, while those refrigerated three months exceeded the controls by twenty times, or 2,000 per cent. They produced lesions in 100 per cent of the inoculated rats after 4 months, whereas those from glycerol at pH 7.5 required 7 months to produce lesions identifiable only at autopsy. The way in which albumin protects infectiousness after prolonged refrigeration is to be considered in a subsequent paper. Meanwhile it may be noted that extraneous substances, not synthesized or burned by the bacilli, can protect them from inhibition *following* animal inoculation and that *in vitro* experiments can be designed which predict the degree of this protection.

Thus, while metabolic study may provide new insight into pathogenesis, the simultaneous inoculation of animals appears necessary in order to learn the basic ground rules. It is only when these rules are understood that metabolic data can replace completely the tedious and expensive observations in animals. Within the general framework reviewed in this paper, this laboratory is prepared to dispense with animal inoculation. In totally new biochemical and biological situations, the animals must again be regarded as a court of final appeal which will dictate the conditions under which metabolic data are acceptable.

The unexpected protection of infectiousness by prolonged soaking in albumin raises the question whether the biochemical content of lipids such as the "cord factor' of the tubercle bacilli (1) must be measured in order to predict infectiousness. The endogenous metabolism of mycobacteria during incubation decreases more rapidly than viability (6). From certain of Bloch's observations on the reduction of methylene blue by tubercle bacilli, it appears that these organisms lose metabolic capacity at a rate which is at least comparable to the burning of lipids and cord factor. The rapid decline of HTC and infectiousness or *M. leprae murium* is too great to be explained by the very slow rates of lipid oxidation. In this case, residual metabolic capacity is more significant than biochemical content. For these reasons it is concluded (*a*) that the effect of aging may be explained by loss of metabolic capacity as readily as by loss of lipids such as cord factor and (*b*) that appropriate measurement of metabolic capacity in incubated mycobacterial suspensions can be accepted as a measure of infectiousness without risk that the results might be invalidated by other factors. Furthermore, these refrigeration studies demonstrate that burning of depletion of special lipids was not a prime determinant of infectiousness. The HTC values, whether increased or

decreased, provided an index of infectiousness except in the special instance of bacilli which had been refrigerated in serum albumin for long periods.

Clearer understanding of the relationship between the physiological state and the infectiousness of mycobacteria is afforded by examining both the tubercle bacilli and *M. leprae murium*. Blóch's observations (1) show, in the first place, that in tubercle bacilli the age of culture is far more important than viable counts as a determinant of infectiousness. In our studies with *M. leprae murium* comparable or greater differences between the infectiousness of fresh and of incubated suspensions have been demonstrated. Similar conclusions have been reached that infectiousness depends on certain special properties in addition to viability (7, 8). Tubercle bacilli in the optimal physiological state exhibit infectiousness by decisive killing of all animals in a group, without latency, chronicity or variable survival times among the animals. Murine leprosy bacilli of high metabolic activity, or when protected from inhibition following inoculation, are also decisively infectious. In short, the mycobacteria either exert a dominance over the host from the earliest moment, or they are placed under physiological restraint from which they do not escape.

As the work progresses, it becomes evident that *metabolic integrity*[7] is the ultimate measure of successful intracellular parasitism or of host resistance. It is only when the metabolic integrity of an infectious agent is at full capacity when it is brought to its final relations with cells, that its enzymatic processes can promptly divert or destroy those of host cells. When the metabolism of the parasite is less completely integrated, dominance is exercised in varying degrees by the host cells. This situation is revealed by failures of infection and by the latency and chronicity so characteristic of mycobacterial infections.

The results obtained with refrigerated tissue homogenates after three months at pH 7.5 (Table 1) are chosen for comparison of the relative delicacy of the HTC and infection methods. In the case of glycerol-refrigerated bacilli, a challenge dose of 0.1 ml of 5 per cent tissue per site required seven months to produce small cutaneous lesions averaging 0.14 gm. each. HTC tests conducted with the equivalent of 1.0 ml of 5 per cent tissue produced 110 μgm. of formazan in four days, or about 11 μgm. per 0.1 ml of sample. Since the HTC recorded would have been tripled by anaerobic incubation for 14 days in the presence of albumin and yeast supplement with 2,000 μgm. of acceptor, and since values as low as 3-5 μgm. can be determined, it is concluded that the metabolic measurement could have been conducted with one-fifth of the number of bacilli included in the skin challenge dose.

[7] In our present concept the term "integrity" indicates completeness of organization and function. It designates resistance to disruption or deterioration of essential enzymatic processes under the conditions in question. To illustrate with an example chosen from observations other than those cited above, *M. phlei* exhibits greater metabolic integrity than other mycobacteria when heated at 60° for 30 minutes, i.e., its metabolism remains integrated and it survives.

Because of the remarkable infectiousness of bacilli after prolonged soaking in purified serum albumin, rat inoculation was in this case a more sensitive indicator. The albumin-refrigerated suspension, if incubated in albumin and yeast supplement with 2,000 μgm. of acceptor for 14 days, should have produced perhaps 500 μgm. of formazan. Although HTC determinations could have been made with 1/100th the number of bacilli used (i.e., one-tenth the number per skin site), it is reasonable that infection could have been established with even smaller numbers. In any event the sensitivity of the HTC method under optimal conditions should permit routine determinations with the numbers of bacilli in our customary skin challenge dose.

The advantages of improved means of preserving murine leprosy bacilli during prolonged intervals between animal experiments or as a safeguard against loss of an animal colony are obvious. Although data from this laboratory indicate that glycerol-refrigerated suspensions are much less infectious than fresh bacilli for moderately resistant rats (7), preservation of infectiousness for more susceptible rats has been reported by Marchoux after 17 months (12), by Linhares after 24 months (11) and by Chorine after 39 months (2) of refrigeration in glycerol.

The best choice of refrigeration menstruum demonstrated in the present work was albumin 15 per cent and yeast supplement 17 per cent (1:6), with maintenance of pH at 7.5. Although the smallest effective concentrations of these reagents has not been determined, other data suggest that lower concentrations may be satisfactory. The capacity for maximal metabolic response was preserved in phosphate buffer as well as in glycerol or sucrose. Any presumed virtues of the latter solutions are open to question. Earlier work has shown that $PO_4$ buffer is as favorable as other electrolytes for preservation of infectiousness during refrigeration (7), and that $PO_4$ buffer at pH 7.5 is one of the optimal electrolytes for sustaining HTC during incubation of washed suspensions (8). The importance of maintaining the pH at 7.5 during prolonged refrigeration suggests that the addition of M/7 or M/15 $PO_4$ to albumin and yeast supplement would be useful.

Although in our experience preservation by refrigeration has distinct advantages, it is not an ideal long-range method. Objections include: the progressive (though very slow) loss of metabolic activity; occasional loss of materials by contamination; the necessity for periodic inspection and pH adjustment; and, particularly, failures of mechanical refrigeration. The recent observations by Eisman *et al* (3) on preservation of *M. leprae murium* at -60 to -70°C are compatible with our experience. In places where dry-ice boxes are in operation, this is the method of choice.

## SUMMARY AND CONCLUSIONS

1. Refrigerated suspensions of *M. leprae murium* have afforded interesting material with which to investigate further the relationships between

metabolic capacity and infectiousness. By inoculating rats with bacilli on which metabolic data were obtained by two methods it has become possible to define more accurately than before the conditions for collecting metabolic data which predict infectiousness. Correlations have been reliable when both respiration and hydrogen transfer capacity (HTC) are modified in the same direction and when metabolic capacity is tested under conditions affording the maximal response which can be induced *in vitro*. In principle these conditions are comparable to those defined in an earlier study of the relationship between the HTC and viability of *M. phlei*.

2. The relationships between different degrees of infectiousness or of host resistance and metabolic integrity of the microorganism have been illustrated and discussed, both with regard to the parasite and the host cell.

3. Examination of the sensitivity of biochemical and infection data revealed that the HTC method can provide significant data when employing the numbers of bacilli contained in our usual skin challenge dose.

4. Both the HTC and infectiousness of *M. leprae murium* during refrigeration at 4-6°C were preserved best at pH 7.5 and in the presence of purified serum albumin and a yeast autolysate. Under those conditions, refrigerated bacilli or infected tissue homogenates should retain useful metabolic activity for fairly long periods of time, and infectiousness for even longer intervals.

## RESÚMEN Y CONCLUSIONES

1. Suspensions refrigeradas de *M. leprae murium* han aportado material interesante para una investigación posterior acerca de la dependencia entre la capacidad metabólica e infectiva. Inoculando ratas con bacilos en los cuales los valores metabólicos han sido obtenidos por dos métodos, se ha podido definir, más exactamente que antes, las condiciones para reunir valores metabólicos que prognostican infecciosidad. La correlación ha sido aceptada cuando ámbos, respiración y capacidad de transferencia del hidrógeno (HTC), cambian en la misma dirección y cuando la capacidad metabólica es examinada bajo condiciones que facilitan la máxima respuesta que puede ser inducida *in vitro*. En principio éstas condiciones son comparables con aquellas expresada en un estudio anterior sobre la "capacidad de transferencia de hidrogeno" y la vitalidad del *M. phlei*.

2. La dependencia de diferentes grados de infecciosidad o resistencia del huésped (mesonero) en relación a la integridad metabólica ha sido expuesta y discutida, ámbos en cuanto al parásito y a la célula huésped.

3. Examinando la sensibilidad de los datos bioquímicos y de infección, encontramos que el método HTC puede proporcionar datos significativos cuando se amplía el número de bacilos contenidos en muestra dosis usual para la prueba cutánea.

4. Ambos, el HTC y la infecciosidad del *M. leprae murium* durante la refrigeración a 4-6°, han sido preservados a un pH de 7.5 y en presencia de sero-albúmina purificada y levadura autolizada. Bajo las condiciones definidas bacilos refrigerados u homogenizados de tejido infectado debe retener actividad metabólica útil por un periodo de tiempo bastante largo y la infecciosidad por un intervalo aún mayor.

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