Volume 45, Number 2
Printed in the U.S.A.

In Vitro Cultivation of Mycobacteria from Human Lepromas and from an Armadillo Infected With *Mycobacterium leprae*[1]

Laszlo Kato and Muhammad Ishaque[2]

Experimental evidence has been presented that the host-grown, hitherto noncultivated mycobacteria, *M. leprae* and *M. lepraemurium* are metabolically competent microorganisms ([1, 5, 6, 8, 11, 12, 14]). They are provided with a complete and functional tricarboxylic acid cycle and electron transport chain. We also described ([5, 6, 10, 11, 13]) that *M. leprae* and *M. lepraemurium* have the capacity to oxidize the following substrates:

1. Sodium succinate.
2. Substances containing SH groups: L-cysteine, penicillamine and thioglucose.
3. Oleic acid and Tween 80.
4. Unidentified components in the heat stable yeast extract (Difco). Oxidation of some of the above substrates resulted in the formation of adenosine triphosphate (ATP) by *M. lepraemurium* ([1]).

Based on the above results, culture media were prepared in which the oxidizable substrates were incorporated as prospective energy sources ([9, 10, 13]). Additional ingredients such as carbon and nitrogen sources were added to the media. The thus prepared culture media were inoculated with *M. leprae* isolated from human lepromas and from leprous tissues of an armadillo. When incubated at 34°C, strongly acid-fast mycobacteria were cultured and regularly subcultured. The cultures of mycobacteria obtained are, so far, unidentified but might be identical to the etiologic agent of human and/or armadillo leprosy.

MATERIALS AND METHODS

Source of host-grown mycobacteria. Suspensions of *M. leprae* were obtained from subcutaneous nodules of untreated lepromatous leprosy cases. Eight nodules were removed aseptically from the skin of eight lepromatous leprosy patients in the Hospital Frei Antonio, Rio de Janeiro, by Dr. Lygia M. Cesar de Andrade. Two subcutaneous lepromas were obtained from Dr. J. Languillon at the Institut de Léprologie Appliquée, Dakar, Senegal from untreated lepromatous cases. This biopsy material reached our laboratories within 14 days, without refrigeration. Three lepromas were removed aseptically at the Department of Dermatology, University of Sao Paulo, Ribeirao Preto, and transported on wet ice to our laboratory within three days. All human tissue samples were collected in 3% glycerol in physiologic saline solution.

Suspensions of acid-fast mycobacteria were isolated from an armadillo (*Dasypus novemcinctus*, L.) inoculated 31 months previously with a pooled suspension of bacilli obtained from lepromatous leprosy skin biopsies. The animal was then killed under sodium amytal anesthesia by bleeding from the heart. Huge skin lepromas together with the spleen and the liver were removed aseptically.

M. leprae inocula were designated as follows:

RP—1, 2, 3 Ribeirao Preto. Untreated LL, Subcutaneous leproma.

JLB—1, 2, 3, 4, 5, 6, 7, 8 Instituto de Leprologia do Brazil. Untreated LL. Subcutaneous lepromas.

JLA—1, 2 Institut de Léprologie Appliquée, Dakar. Untreated LL. Subcutaneous lepromas.

A5 sc. Armadillo injected with *M. leprae*. Subcutaneous leproma. ALM Leprosy Atelier, University of Hawaii.

A6 sc. Same.

A6 sp. Same, spleen.

A6 li. Same, liver.

A6 pooled. Same, skin, spleen and liver.

Small samples of the human and armadillo tissues were homogenized in a glass Potter

[1] Received for publication 19 May 1977.

[2] L. Kato, M.D., Professor and Head; M. Ishaque, Ph.D., Associate Professor, Hansen Research, Chair of Bacteriology Research Center, Institut Armand-Frappier, University of Quebec, P. Que., Canada.

homogenizer and suspended in potassium buffer solution (pH 5.8) in approximately 20 times the volume of the tissues. These suspensions were centrifuged at 2,000 rpm for two minutes and the sediment was discarded. The supernatant was centrifuged for ten minutes at 8,000 rpm at +4°C. The sediments obtained, extremely rich in acid-fast bacilli, were resuspended in the phosphate buffer solution and inoculated into the media.

The remaining suspension of bacilli was centrifuged again at 8,000 rpm for ten minutes. The supernatant was discarded and the sediment was resuspended in NaOH-citrate solution. In order to eliminate secondary contaminants other than mycobacteria in the tissue homogenate, the bacilli were exposed to NaOH-citrate treatment for 20 minutes. The suspension was centrifuged again for 8,000 rpm for ten minutes and the sediment was washed three times with the phosphate buffer solution. The decontaminated sediment was then resuspended in the phosphate buffer and inoculated into the culture media.

The huge subcutaneous lepromas and the spleen and liver of the armadillo were kept on wet ice for 42 hours. The pooled tissues weighed 162 gm. The tissues were pooled, chopped with scissors and washed with cold distilled water. They were then homogenized in the phosphate buffer solution in a Sorvall Omnimixer and the homogenate was filtered through a sterile nylon filter. The filtrate was centrifuged at +4°C, 8,000 rpm for eight minutes. The supernatant was carefully decanted and the sediment was resuspended and washed four times. The combined suspensions were centrifuged at 10,000 rpm for ten minutes and the sediments washed twice in cold buffer. The fluffy superficial layer above the sedimented bacilli was pulled off and the liquid sticking on the side of the tube was washed off. The sediment was resuspended in phosphate buffer and this cell suspension was used as inoculum in the culture media.

The phosphate buffer contained 8.2 gm KH_2PO_4 and 0.5 gm of Na_2HPO_4 in one liter distilled water. These solutions had a pH of 5.8.

The NaOH-citrate solution was freshly prepared. NaOH 4% in distilled water and sodium citrate 2.9% in distilled water were mixed in equal volumes. The culture medium, KI-1, contained 2 gm yeast extract (Difco) and 30 gm glycerol in one liter phosphate buffer.

The medium was distributed into 50 ml screw cap tubes. Each tube contained 9 ml of the liquid medium. The liquid medium was sterilized in an autoclave at 15 lb for 20 minutes.

Two hundred units of penicillin G-sodium was added to all of the media prior to inoculation.

Sheep serum was sterilized by filtration. One milliliter of the sterile serum solution was added to each tube containing 9 ml of the media prior to inoculation with the bacilli.

The carefully homogenized suspensions of mycobacteria isolated from human or armadillo lepromas were inoculated into the liquid culture media under aseptic conditions. The inoculum was suspended and diluted in phosphate buffer so that the inoculated liquid media contained a relatively high number of acid-fast bacilli. The concentration of the acid-fast bacilli in the media was determined as a relative base line 8 to 12 hours after inoculation.

Criteria for growth and multiplication. A slight or incipient growth of acid-fast bacilli in the media was not considered to be achieving *in vitro* cultivation of the host-isolated mycobacteria. Four to twelve hours after the media were inoculated, the base line was established, estimating the relative density of the inoculum in the liquid media. For this purpose the culture was vibrated on a shaker for five to ten seconds to achieve homogenization of the inoculum in the medium. This was done not earlier than four hours after inoculation of the media. A loopful of the inoculated medium, using a 2 mm platinum loop, was placed on a slide and spread evenly over a 1 cm diameter surface. The preparation was dried in a paraffin oven at 60°C and fixed by flaming. Since young cultures of such mycobacteria are usually non-acid-fast, easily decolorized and often not detected with conventional technics, the preparations were treated with periodic acid solution to reveal those forms of mycobacteria which would remain otherwise undetected. A 5% to 10% aqueous solution of periodic acid was poured over the slide and heated by flame until the first signs of evaporation became visible. Two minutes later the preparations were washed with tap water and stained by the Ziehl-Neelsen method. The number of

TABLE 1. *Results.*

Inocula	Human (H) Armadillo (A)	NaOH treated	Days from biopsy to medium	Transport temp.	Transport medium	Primary culture: Incipient growth estimated ± days	Primary culture: Heavy growth ± days	Heavy growth ± days in subcultures: 1st	Heavy growth ± days in subcultures: 2nd
RP—1	H	+	3	ice	g-w	76	30	20	14
RP—2	H	+	3	ice	g-w	20	36	21	14
RP—3	H	+	4	ice	g-w	20	36	21	14
JLB—1	H	+	12	ambiante	g-w	60	90	30	14
JLB—5	H	+	12	"	g-w	60	90	30	14
JLB—7	H	+	12	"	g-w	60	90	30	14
JLB—2, 3,4,5,6,8	H	0	12	"	g-w	con-taminated			
JLA—2	H	+	14	"	g-w	20	60	21	20
JLA—3	H	0	14	"	g-w	90	120	28	20
A5—Sc	A	+	45	+4°C	hyal.ac.				
A6—Sc	A	+	0.2	"	—	18	25	20	14
A6—Sc	A	0	0.2	"	—	18	30	20	10
A6—Sp	A	+	0.2	"	—	18	30	20	14
A6—Sp	A	0	0.2	"	—	18	30	20	14
A6—Li	A	+	0.2	"	—	24	46		
A6—Li	A	0	0.2	"	—	24	36	20	16
A6—Sc. spli.	A	+	3	ice	PO_4	12	21	15	10
A6—Sc. spli.	A	0	3	ice	PO_4	12	21	15	10

bacilli per microscopic field, scanning diagonally on the 1 cm diameter preparation was determined. The average number per field was set as a base line for evaluation of cultivation progress. The base line was usually 5 to 50 bacilli per field depending on the size of the inoculum. The progress of cultivation was estimated by the appearance of a sediment or turbidity in the liquid medium and microscopically by the increase in the number of bacilli in the stained preparations. Again for practical purposes, the progress of cultivation was estimated by two parameters:

1. Estimation of approximate time in days when visible growth occurred visually and microscopically. This was the approximate time in days when sediment or turbidity developed in the liquid media and when the number of bacilli per field in the stained preparation left no doubt that the number of bacilli per field increased at least twice or four times relative to the base line.

2. Approximate time in days after inoculation when heavy sediment or turbidity developed in the media and microscopic examination of the stained preparation revealed that the number of bacilli per preparation was so heavy as to preclude the counting of the acid-fast units.

By using these simple parameters, there is no doubt in the mind of trained microbiologists that positive cultures of a mycobacterial strain progressed in a logarithmic manner.

RESULTS

Results were summarized schematically in Table 1.

Whenever growth occurred it was initially slow and dependent on the size and source of inoculum. When bacilli were separated from the tissues and inoculated into the media within hours or just a few days after biopsy or autopsy, an incipient growth was observed within 10 to 20 days in the primary culture. When specimens were transported in glycerol-water solution for a period of 10 to 15 days without refrigeration, they often arrived in our laboratories as partially autolyzed tissues and often more or less contaminated. Bacilli isolated from such tissues were inoculated into culture media after decontamination with NaOH citrate solution. From such inoculated media the incipient growth was observed after a period of five to ten weeks. The appearance of the initial slow growth was independent of whether or not the specimens were treated with NaOH, however a relatively heavy inoculum was necessary to achieve growth. Once the incipient growth appeared as a precipitate in the medium or a turbidity after shaking the cultures, a heavy bacillary precipitate usually developed within an additional two weeks. Secondary inoculations into the homologue medium resulted with every subculture in a faster growth than in the primary cultures. In the second and third subcultures, heavy bacillary precipitate developed within 8 to 20 days depending on the size of the inoculum. The precipitate was colorless or slightly yellowish in the primary culture, but became more and more scotochromogenic in further subcultures. The bacillary precipitate was finely granular but resulted in a seemingly homogeneous turbidity when shaken vigorously. However, partial or complete sedimentation occurred within 24 hours after incubation at 34°C.

The growth morphology. In the young slow-growing cultures most of the bacilli are non-acid-fast with ordinary Ziehl-Neelsen technics. After oxidation with periodic acid, the acid-fastness is restored even in the young cultures. In the young growing cultures, the individual or clumped bacilli are seen as short rods. With the cultivation progressing to a heavy growth in the media, the majority of the bacilli are short, strongly acid-fast rods. A few bacilli are unusually long and even have a filamentous shape. In the secondary cultures when growth is more rapid, the proportion of acid-fast bacilli predominates even in the young cultures. In the primary as well as the secondary cultures bacilli tend to agglomerate as clumps in the young cultures consisting of just a few bacilli. As growth progresses, the clumps increase in size and even after vigorous shaking the clumps are formed by hundreds of bacilli, unable to be counted under the microscope. Bacilli have a typical arrangement in the clump which resembles the random arrangement in the "globi."

Extensively slow primary and subcultures are grown on the surface of solid KI-1 media, prepared with 3% agar, as small, round, glossy, yellowish scotochromogenic colonies. Growth characteristics on solid media will be described elsewhere.

Bacilli isolated from human or armadillo leprous tissues obtained by biopsy or autopsy were inoculated into Dubos liquid and Lowenstein-Jensen media. No growth or multiplication occurred during the eight week observation period at 34°C. Expecting that cultures, once adapted to extracellular life, would later grow on conventional culture media used for mycobacteria, the further subcultures were regularly inoculated into Lowenstein-Jensen and Dubos media and observed for growth. Adaptation to grow on these substrates can be expected ([7, 10, 13]), and pertinent observations will be reported later.

Identification and classification of the obtained cultures are now under investigation.

DISCUSSION

Hanks expressed the view in 1954 ([2, 3]) that, "Until the problem of . . . utilization of exogenous substrates *in vitro* has been demonstrated, it would appear that direct bacteriological attempts to cultivate human and murine leprosy bacilli will continue to be unproductive." This opinion has often been expressed and recently Pattyn ([17]) wrote, "One might hope that a better understanding of the biochemistry of the leprosy bacillus might have solved the problem of cultivation." Few data are available on the metabolism of noncultivable host-grown microorganisms. *M. leprae* and *M. lepraemurium* are generally considered as host-dependent intracellular parasites ([2-4]). Opinions have been expressed to the effect that these noncultivable microorganisms are metabolically deficient with an incomplete tricarboxylic acid cycle and electron transport

chain lacking in cytochrome-c ([15, 16]). Using more sophisticated methodology, it became evident that host-grown human and murine leprosy bacilli are metabolically competent with a functional and complete tricarboxylic acid cycle and electron transport chain ([5, 6, 8-12, 14, 18]).

Multiplication of bacilli in the host or in artificial media are energy dependent biological processes. For growth, multiplication and virulence, microorganisms are not too selective as to the source of carbon or nitrogen. However, most specific substrates are enzymatically oxidized by bacteria in order to derive energy by direct substrate oxidation or via oxidative phosphorylation. Hanks and Gray ([4]) were the first to search systematically for substrates of oxidizable energy sources by these elusive microorganisms. They concluded that leprosy bacilli are unable to burn such substrates which cultivable mycobacteria can oxidize to derive energy for growth, multiplication and virulence. During the past 26 years in our effort to cultivate *M. leprae* and *M. lepraemurium,* we searched systematically for substrates oxidizable by host-grown mycobacteria. Using manometric methods we recently described a series of substrates which are oxidizable by *M. lepraemurium* and some of them by *M. leprae* ([5, 6, 10, 11, 13]). On culture media which contained yeast extract, succinate or L-cysteine respectively, as oxidizable energy sources for host-grown mycobacteria, we regularly obtained cultures of mycobacteria from rat lepromas which were easily and abundantly subcultured on the homologue media ([10]). The same strains did not grow on conventional culture media used for the cultivation of mycobacteria. However, after several subcultures they were adapted and easily grown on Lowenstein-Jensen solid medium. These cultures were identifiable as scotochromogenic mycobacteria belonging to the group of scrofulacea ([10, 11]). Skinsnes and co-workers ([18]) cultured several strains of mycobacteria from leprous tissues on a hyaluronic acid-based medium and claimed the successful cultivation of *M. leprae.* These cultures later became adapted to Lowenstein-Jensen medium and were identified as *M. scrofulaceum* ([11]). According to Pattyn (in [11]) the cultures described by Skinsnes are not identical with the etiologic agent of leprosy. In agreement with Pattyn, Kato ([11]) recognized the Honolulu strain as *M. scrofulaceum,* however he came to the conclusion that *in vitro* grown *M. leprae* might be a member of the *M. scrofulaceum* species. With our present state of incomplete knowledge, the cultures isolated by the Skinsnes group and our team from human lepromas as well as the numerous cultures of mycobacteria cultured from murine leprotic tissues, still await final identification and classification.

Our present results show that from human and armadillo leprous tissues, hitherto unidentified strains of mycobacteria can be cultured and subcultured abundantly on media which contain substrates oxidizable by host-grown mycobacteria. The biopsy or autopsy specimens can be treated with NaOH for decontamination from secondary contaminants. Treatment of the specimens with NaOH still permits the cultivation of mycobacteria from the thus treated specimens. It seems that the specimens can be transported for a period of up to 14 days in glycerol-water solution. However, the sooner the bacilli are isolated from the specimens and inoculated into the media, the higher the chance for successful cultivation of the described cultures, provided a heavy inoculum is used.

Further investigations are necessary to establish the identity and the probable role or the eventual relation of the obtained cultures to the etiology of armadillo and/or human leprosy.

SUMMARY

M. leprae was isolated by partial purification from human lepromas as well as from skin nodules, spleen and liver from an armadillo injected 31 months previously with *M. leprae* from pooled bacillary isolates from several lepromatous patients. The medium (KI-1) contained in one liter of distilled water: KH_2PO_4—8.2 gm; Na_2HPO_4—0.5 gm; yeast extract "Difco"—2 gm; and glycerol—30 gm. Nine milliliters of the medium were distributed into 50 ml screw cap tubes and autoclaved for 30 minutes. To each of the tubes 1 ml sterile filtered sheep serum and 200 units per ml penicillin G-sodium were added. In order to obtain the primary culture a relatively heavy inoculum was used. Incubation temperature was 34°C. From human cutaneous lepromas, growth of acid-fast bacilli was observed within two

weeks in the primary cultures when inoculated not later than three days after biopsy. When media were inoculated with bacilli from lepromas shipped without refrigeration for more than 14 days, there was either no growth in the media or the incipient multiplication occurred only after six to eight weeks. In most cases the isolated bacilli from the tissues were decontaminated with a solution containing equal volumes of 4% sodium hydroxide and 2.9% sodium citrate. Such treatment for decontamination of secondary contaminants did not alter the success of cultivation when bacilli were exposed for 20 minutes to the decontamination treatment and then washed three times with abundant amounts of phosphate buffer.

In the young cultures, bacilli were non-acid-fast and their presence was revealed only by staining after periodic acid oxidation. Once the incipient growth was observed, abundant multiplication developed in the media within two to three weeks. This was visible as abundant finely granular sediment in the liquid media and strongly acid-fast rods in clumps when examined microscopically. The cultures of mycobacteria were easily subcultured when transferred into the homologue media. Depending on the size of the inocula, the subcultures still grow as slow growers but faster than the primary culture.

Though many characteristics of the cultures are already known, their relation to *M. leprae* or to the etiology of leprosy awaits verification.

RESUMEN

Se infectaron armadillos con suspensiones del *M. leprae* aislado de varios pacientes lepromatosos. Se prepararon suspensiones parcialmente purificadas del *M. leprae* a partir de lepromas humanos así como de los nódulos de la piel, del bazo y del hígado de un armadillo con 31 meses de infección. El medio (KI-1) contenía en 1 litro de agua destilada, 8.2 gm de KH_2PO_4, 0.5 gm de Na_2HPO_4, 2.0 gm de extracto de levadura "Difco," y 30 gm de glicerol. El medio se distribuyó en volumenes de 9.0 ml en tubos de 50 cc con tapón de rosca y se esterilizó en el autoclave durante 30 min. A cada tubo se le adicionó 1.0 ml de suero estéril de carnero y 200 unidades por ml de penicilina G sódica. Hubo necesidad de utilizar un inóculo relativamente grande para lograr el cultivo primario. La temperatura de incubación fue de 34° C. En el caso de los lepromas cutáneos humanos el crecimiento de bacilos resistentes al alcohol ácido se observó dentro de las primeras 2 semanas del cultivo primario cuando las inoculaciones se hicieron dentro de los 3 días siguientes a la toma de la biopsia. Cuando los medios se inocularon con bacilos de lepromas mantenidos sin refrigeración por más de 14 días, no hubo crecimiento o éste comenzó en forma incipiente después de 6 a 8 semanas. En la mayoría de los casos los bacilos aislados de los tejidos fueron descontaminados con una solución preparada con partes iguales de hidróxido de sodio al 4% y citrato de sodio al 2.9%. Este tratamiento no alteró el éxito del cultivo cuando los bacilos se expusieron a la mezcla descontaminante durante 20 min y después se lavaron 3 veces con una abundante cantidad de regulador de fosfatos.

En los cultivos jóvenes, los bacilos no fueron resistentes al alcohol ácido y sólo se pudieron hacer evidentes por tinción después de oxidación con ácido peryódico. Una vez que se observó el crecimiento incipiente se desarrolló una abundante crecimiento durante las 2 ó 3 semanas siguientes. Esto fue visible en la forma de un abundante crecimiento finamente granular en el medio líquido. Microscopicamente se observaron grumos de bacilos resistentes al alcohol ácido intensamente teñidos. Los cultivos de estas micobacterias se subcultivaron facilmente en el medio homólogo fresco. Dependiendo del tamaño del inóculo, los subcultivos aún crecieron en forma lenta pero más rápido que los cultivos primarios.

Aunque ya se conocen muchas caracteristicas de los cultivos, su relación con *M. leprae*, o con la etiología de la lepra aún está en espera de ser verificada.

RÉSUMÉ

Des suspensions partiellement purifiées de *Mycobacterium leprae* furent préparées à partir de lépromes humains, ainsi qu'à partir de nodules cutanées, de la rate et du foie d'un tatou injecté 31 mois auparavant avec *Mycobacterium leprae* provenant d'un patient atteint de lèpre lépromateuse. Le milieu de culture (KI-1) contenait par litre d'eau distillée: KH_2PO_4—8.2g.; Na_2HPO_4—0.5g.; extrait de levure "Difco"—2g.; glycérol—30ml. Neuf ml de ce milieu est distribué par éprouvette à pas de vis de 50 ml, puis autoclavé 30 minutes. A chaque éprouvette, on ajoute un ml de sérum de mouton stérilisé par filtration et de la pénicilline G sodique à une concentration finale de 200 U.I. par ml. Afin d'obtenir la primo-culture, l'inoculum utilisé est assez considérable. La température d'incubation est de 34°C. Dans les cultures à partir de lépromes humains, la croissance de bacilles acido-résistants est observée en deux semaines dans les primo-cultures lorsque les milieux étaient inoculés pas plus tard que la troisième journée suivant la biopsie. Lors-

qu'on inoculait les milieux avec du matériel expédié sans réfrigération, vieux de 14 jours, il y a soit aucune croissance ou bien la multiplication n'était évidente qu'après six à huit semaines. Dans la plupart des cas, les bacilles isolés des tissus furent décontaminés avec une solution obtenue en mélangeant des volumes égales de solutions de NaOH—4% et de Na_3 citrate—2.9%. Un tel traitement de décontamination n'altérait aucunement les chances de succès quant à la culture des bacilles, lorsque traités pour 20 minutes puis lavés trois fois avec une grande quantité de tampon phosphate.

Chez les cultures jeunes, les bacilles n'étaient pas acido-résistants et leur présence devenait apparente uniquement après une oxydation à l'acide périodique. Dès que la croissance est amorcée, on obtient une bonne poussée en deux à trois semaines. On observe un abondant sédiment finement granulaire en milieu liquide, et au microscope il s'agit d'amas bacillaires acido-résistants. Les cultures sont aisément sous-cultivables sur milieu homologue. Ces sous-cultures poussent lentement, mais plus rapidement que la primo-culture.

Bien que plusieurs caractéristiques de ces cultures sont déjà connues, leur relation à *M. leprae* ou à l'étiologie de la lèpre doit être vérifiée.

Acknowledgments. These investigations were supported by the "Institut Fame-Pereo" and "La Fondation Merieux" (Lyon, France). The Hansen Chair of Research is supported by "The Leprosy Relief (Canada) Inc." Biopsy material was kindly supplied by Dr. Lygia M. Cesar de Andrade, Instituto de Leprologia de Brazil, Rio de Janeiro; by Dr. J. Languillon, Institut de Léprologie Appliquée, Dakar, Senegal; by Dr. L. M. Bechelli, University of Sao Paulo, Ribeirao Preto; and by Dr. Olaf K. Skinsnes, ALM Leprosy Atelier, University of Hawaii. The continual cooperation of these colleagues is greatly appreciated.

REFERENCES

1. ADAPOE, C. Respiration, electron transport and ATP formation in a noncultivated mycobacterium. Ph.D. Dissertation, University of Montreal, Canada, 1976.
2. HANKS, J. H. Influence of physical and chemical factors on the hydrogen transfer capacity of murine leprosy bacilli. Int. J. Lepr. **22** (1954) 162-173.
3. HANKS, J. H. Relationship between the metabolic capacity and the infectiousness of *Mycobacterium lepraemurium:* refrigeration studies. Int. J. Lepr. **22** (1954) 450-460.
4. HANKS, J. H. and GRAY, C. T. The application of metabolic studies to leprosy research. Int. J. Lepr. **22** (1954) 147-161.
5. ISHAQUE, M. and KATO, L. Oxidation of various substrates by host-grown *Mycobacterium leprae* and *Mycobacterium lepraemurium*. Rev. Can. Biol. In press.
6. ISHAQUE, M., KATO, L. and SKINSNES, O. K. Cytochrome linked respiration in host-grown *Mycobacterium leprae* isolated from an armadillo (*Dasypus novemcinctus,* L.) Int. J. Lepr. **45** (1977) 114-119.
7. KATO, L. *In vitro* grown *M. leprae* probably a member of the *M. scrofulaceum* species. Int. J. Lepr. **44** (1976) 385-386.
8. KATO, L., ADAPOE, C. and ISHAQUE, M. Respiratory metabolism of *Mycobacterium lepraemurium*. Can. J. Microbiol. **22** (1976) 1293-1299.
9. KATO, L. and ISHAQUE, M. A simplified hyaluronic acid based culture medium for mycobacteria isolated from human lepromata. Int. J. Lepr. **44** (1976) 431-434.
10. KATO, L. and ISHAQUE, M. Culture et infectivité des mycobactéries scotochromogènes isolées à partir de tissus lépreux de rats. Acta Leprol. In press.
11. KATO, L. and ISHAQUE, M. Mycobacteria cultivated on a hyaluronic acid-based medium from leprous tissues of an armadillo. In press.
12. KATO, L. and ISHAQUE, M. Oxidation of reduced nicotinamide adenine dinucleotide by particles from *Mycobacterium lepraemurium*. Cytobios. **12** (1975) 31-43.
13. ISHAQUE, M. and KATO, L. Oxidation of substrates by host-grown *Mycobacterium leprae* and *Mycobacterium lepraemurium* and by *in vitro* grown mycobacteria cultured from human and murine lepromata. Int. J. Lepr. **45** (1977) 120-131.
14. KATO, L., ISHAQUE, M. and WALSH, G. P. Cytochrome pigments in *M. leprae* isolated from armadillos. Microbios. **12** (1975) 41-50.
15. MORI, T., KOHSAKA, K. and DOHMAE, K. Terminal electron transport system of *M. lepraemurium*. Int. J. Lepr. **39** (1971) 813-828.
16. MORI, T., KOHSAKA, K. and TANAKA, J. Tricarboxylic acid cycle in *M. lepraemurium*. Int. J. Lepr. **39** (1971) 796-812.
17. PATTYN, S. R. The problem of cultivation of *Mycobacterium leprae*. Bull. WHO **49** (1973) 403-410.
18. SKINSNES, O. K., MATSUO, E., CHANG, P. H. C. and ANDERSSON, B. *In vitro* cultivation of leprosy bacilli on hyaluronic acid-based medium. Int. J. Lepr. **43** (1975) 193-209.