WATER SOLUBLE PALMITIC ACID IN MEDIA FOR CULTIVATION OF LEPROSY DERIVED PSYCHROPHILIC MYCOBACTERIA FROM *MYCOBACTERIUM LEPRAE* INFECTED TISSUES

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1. INTRODUÇÃO

A unique marker of *Mycobacterium (M) leprae* is that it cannot derive energy, use carbon sources, or substrates for biosynthetic purposes, whereas other mycobacteria utilize these for multiplication. This is just one reason why *M. leprae,* although provided with a complete Krebs cycle, a functional electron transport chain and cytochrome system, as well as measurable endogenous respiration, does not grow in any of the tested culture media. Yet it multiplies abundantly in tissues of susceptible hosts. For the above reasons, *M. leprae* is a most unusual and unique microbe. Cultivation trials under most conditions have therefore been proposed repeatedly in our laboratories over four decades. Media were tried unsuccessfully with extreme pH and ionic strength, with rich and poor media incorporating polysaccharides, steroids, hydrocarbons, a catalogue of prospective nutrients, even parabiotic cultures. The frequent proposal of new variations of media motivated by consistent failure to achieve cultivation. The unusual conditions and compositions of media were not some acrobatic exercise, but a follow-up of a research philosophy that a highly unusual microbe must grow under most unusual conditions. Why then should one persist with the same conventional media wich do not work?

Following our leitmotiv in cultivation trials, we obtained a flock of cultures. These strains were identical to the now well-known leprosy derived cultivable mycobacteria (LDM) (14). some of them resembled *M. leprae* or were hard to identify, but only in the early subcultures. These were the mixed cultures, none of them the authentic *tic M. leprae.*

Recently this author reported the cultivation of several strains of a hitherto unknown cluster of cold living mycobacteria from *M. leprae* infected tissues[13]. Tentatively these were named *M. psychrophilum* (L). Caustiously the successful cultivation of *M. leprae* was not claimed, although the cultures were regularly isolated from leprosy infected tissues. The cultures do not grow on media for cultivable mycobacteria, except on a multifactorial medium optimally at + 10°C.

The recently discovered preparation of heat stable, water soluble palmitic acid and palmitates by Kato, Szetjli and Szente[16] permitted the development of chemically well defined new culture media for easily reproducible growth of the leprosy derived *M. psychrophilum* (L).

The purpose of the present investigation was to satisfy the author's curiosity as to whether a hitherto unknown psychrophilic cluster of mycobacteria really existed, rather than to identify the cultures as being *M. leprae* or not.

Progress will be reported in this communication.

2. MATERIALS AND METHODS

Multifactorial medium. Ammonium thioglycolate served as the sole source of N with and SH group as a prospective source of energy[9,12.] Water soluble palmitic acid and its salts were used as potent energy sources[6,10,13]

Liquid medium. The chemically well defined medium was prepared as follows.

Solution A. In 900 ml distilled water were dissolved by magnetic stirring $KH_2PO_„$ 4g;

Na_2HPO_4, 2 g; $MgSO_4$, 0.05 g; ferric ammonium citrate 0,05 g with 0.1 g b cyclodextrin.

Solution B. Water soluble palmitic acid, Na palmitate or ascorbic palmitate was used[1]s. Palmitic acid or its salts; 300 mg was placed in a mortar and with constant grinding slowly cold distilled water was added till a crystal clear solution was obtaneid. A total of 80 ml water was slowly mixed to the clear solution during not less than 3 min, to avoid physical displacement of the fatty acid from the inclusion complex by molecular H_20.

With constant vigorous magnetic stirring 20 ml ammonium thioglycolate (Fluka, 60% w/vin H_20) and Solution B were mixed to Solution A. These manipulations were done under a hood or in a well ventilated area.

The final solution was a bluish color with a pH 6.0-6.2. Anyfurther adjustment of the pH was strictly avoided to prevent precipitate formation during sterilization in the autoclave. The medium was distributed 20 mV50 ml screw cap tube and autoclaved for 25 min.

Semisolid medium. Liquid medium was prepared without the addition of ammonium thioglycolate. To 1 L liquid medium 30 g granulated agar was mixed by magnetic stirring. The agar suspension was dissolved by heating to 90100° in a water bath or autoclave. The solution was cooled to 60-70° and with vigorous magnetic stirring 20 ml ammonium thioglycolate was slowly poured into the hot solution.

The medium thus prepared was distributed while hot, 20 mV50 ml screw cap tube, autoclaved for 25 min cooled in an inclined position to make agar slants.

Cultures. *Mycobacterium leprae* semipurified suspension was prepared as previously described° from an *M. leprae* infected armadillo nodule, from Nu mice foot pads or from human lepromata. The tissues were washed with sterille distilled water and cut aseptically with scissors into small pieces. Using a4 blade Lourdes Model MM1b homogenizer, the tissues were homogenized 4 times for 5 seconds at maximal speed with cold distilled water, 10 times the weight of the tissue (v/w). The suspension was filtered through a cca 20 x 20 cm nylon tissue (cut from nylon stockings) and fastened over a 250 ml beaker, then autoclaved for 30 min. The filtered cell suspension was centrifuged at 6,000 rpm for 10 min. The sediment was washed twice with distilled water. The use of sterile distilled water instead of buffers of physiological saline resulted in lysis of blood cells and prevented sweling of tissue muco-polysaccharides. The semipurified suspension of *M. leprae* cells was adequately separated from undesirable impurities, forcultivation trials, but not for metabolic studies. The sediment so washed suspended for decontamination (18) in excess of 1% o benzalkonium chloride (Zephiran) in 5% Na_2PO_4 solution in sterile distilled water, followed by constant slow magnetic stirring for 50 min at room temperature. The suspension was again centrifuged at 6,000 rpm for 10 min. The sediment was washed once with the sterile liquid medium. Cells were separated by centrifugation and the thick paste obtained was used as heavy inoculum to the semisolid media. Liquid media were inoculated out of a diluted suspension of *M. leprae,* so as to obtain an average of 25-50 acid fast cells per microscopical field, as prepared on slides for estimation of growth.

M. avium, M. intracellulare and M. scrofulaceum were isolated from *M. leprae* infected armadillo tissues.

M. phlei was isolated from a human leproma.

The above cultures were stored, freeze dried, as leprosy derives mycobacteria (LDM).

The LDM were transferred from storage into Middlebrook 7H9 liquid media and incubated at 36°. The centrifuged, washed 10 day old LDM cultures were inoculated into liquid and solid multifactorial media and liquid 7H9 media. The cultures were incubated at +10, +22, and +32°C respectively.

Media were also inoculated with autoclaved suspensions of *M. leprae.* These served as controls.

Cold Temperature Incubator. In a simple, regular, used refrigerator, thethermoregulator switch was replaced by thermoregulator to maintain a constant temperature of +10° (+- 1°C) inside the 9 cubic foot space.

Estimation of growth in media.

Since the palmitates and ammonium thioglycolate in the medium have a detergent

effect, the cells are easily washed off from normal slides. Furthermore, the growth developed in huge clumps, impossible to disperse even with vigorous mechanical shaking. A special procedure was therefore necessary to visualize and quantitate growth kinetics.

Following 10 sec shaking with a Vortex mixer, 1 ml of the culture was withdrawn with sterile syringe and needle. This amountwastransferred into a rubberstoppered glass tube (100 x 16 mm). Ten ml distilled water was pipetted into the tube, and thoroughly mixed. Cells were separated by 10 min centrifugation at 3,000 rpm. The supernatant was withdrawn with syringe attached to 10 cm needle. The sediment was suspended in 1 ml distilled water and shaken for 10 sec with a Vortex mixer. Added 1-2 drops of chloroform was used for declumping during 10 sec shaking with Vortex mixer.

A 5 mm loopful of the so prepared declumped suspension or its dilutions was evenly spread over a 10 mm diameter surface of a siliconized slide. Slides were left at room temperature overnight for drying. Fixation over flame was followed by Ziehl-Neelsen staining and 15 sec decolorization with 3% Hcl in 75% ethanol. Average number of cells in 20 microscopical fields was registered at 1000x magnification, taking into account the dilutions of the cultures.

Growth on the semisolid agar media was easily detected by increased bacterial mass and colony formation on the surface, as compared to the control tubes inoculated with heat killed M. *leprae* cells.

Percentage of solidly stained acid fast rods was counted microscopically on siliconized slides following Ziehl-Neelsen staining of thin smears of cell suspensions after declumping with chloroform.

3. RESULTS

Results presented in Tables 1 and 2 are only relative values, but express fairly the multiplication of inoculated acid fast rods. Counting of bacilli in stained preparations was difficult because the number of cells washed of from the slides during staining could not be estimated. Furthermore, growth occurred as some huge clumps, that did not disintegrate following declumping with chloroform. The growth however was so obvious for the trained eyes of students of bacteriology, that the microscopical readings offered acceptable information concerning growth of the cultures.

Results in Table 1 show multiplication of inoculated acid fast rods at 10°C during 10 weeks of incubation in the liquid media. No growth was observed at+22 and at +32°C during the observation period. At 10°C incubation the morphology of cells was not only well preserved, but improved with multiplication in progress. By contrast, at 22 and 32°C incubation, cellular morphology rapidly deteriorated after 2-4 weeks of incubation. Table 1 shows results obtained on the primary cultures in media inoculated from subcutanous lepromata of an armadillo (A7) and cells collected from foot pads of *M. leprae* infected Nu mice (M3). The table also showsthat multiplication at 10°C started before the 2nd week after inoculation of the armadillo derived bacilli, but about a week earlier in media inoculated with cells from foot pads of Nu mice.

Heat killed A7 and M3 cell suspensions did not grow in the sodium palmitate liquid media. Acid fast cell suspensions from A7 and M3 lepromata did not grow on Loewenstein or in 71-19 or Dubos liquid media at 10, 22 and 32°C.

Results presented in Table 1 indicate that maximal growth was probably achieved at 8 to 10 weeks of incubation. Cultures were therefore transferred into fresh homologous media at that time as a 1:10 dilution.

Table 2 shows schematic representation of growth of *M. psychrophilum (L)* in the primary culture as well as in several subcultures. Inoculated cells of acid fast bacilli were separated from an armadillo (A6) and a human (H4) leproma, grown in sodium palmitate liquid media at +10°C. It took 2 to 4 weeks of incubation before multiplication of cells occurred in the primary cultures. This latency period of growth was considerably shortened in the subcultures. These results are a clear indication of the adaptation of host grown cells to the chemical and physical conditions for *in vitro* growth.

A6, A7, H4 and M3 derived authentic host grown M. *leprae* cell suspensions from the

KATO, L Water soluble palmitic In media for cultivation of leprosy derived psychrophilic my mycobateda from *Mycobacterium Leprae* infected tissues.

TABLE 1 - Growth of *Mycobacterium psychrophilum* (L) at different incubation temperatures in sodium palmitate liquid media. Cultures were Isolated from an *M. leprae* infected armadillo (A7) and foot pads of Nu mice (M3).

		Incubation (Time in Weeks)					
Culture	Temp. °C	0	1	2	4	8	10
A7	10	+	+	+++	++++	>++++	++++
	22	+	+	+-	+-	+-	+-
	32	+	+-	+-			
M3	10	+	+++	++++	>++++	>++++	>++++
	22	+	+	+-	+-	+-	+-
	32	+	+	+-			

Acid fast bacilli per microscopical field; +, 20-40; ++, 50-75; +++, 100-200; ++++, > 200; >++++, heavy growth. +-, deteriorating morphology.

TABLE 2 - Growth of *Mycobacterium psychrophilum* (L) in sodium palmitate liquid media primary cultures and subcultures and subcultures at 10°C Incubation temperature. Cultures were isolated from an armadillo (A6) and human H4) subcutaneous lepromata.

		Transfer	Weeks	Incubation (10° C)			
Strain	Cultures	Times in Weeks	0	1	2	4	8
Primary	A6	0	+	+	++	++++	>++++
1st sub.		8	+	+	+++	>++++	
4th sub.		8	+	++	++++	>++++	
7th sub.		8	+	+++	++++	>++++	
Primary	H4	0	+	+	+	1-++	++++
1st sub.		12	+	++	++	++++	
2nd sub.		8	+	++	+++	++++	
3rd sub.		8	+	++	+++	++++	

Acid fast bacilli per microscopical field; +, 20-40; ++, 50-75; +++, 100-200; ++++, > 200; >++++, heavy growth. +-, deteriorating morphology.

corresponding lepromata were also inoculated into liquid media prepared with the same concentrations of water soluble palmitic acid, Na palmitate and ascorbic palmitate. Results not presented in the tables left no doubt that multiplication of acid fast rods was practically the same with any of the water soluble palmitic acid salts.

The inoculum from authentic *M. leprae* suspensions isolated from human (H), nude mice (M) or armadillo (A) lepromata, as well as the subcultures of *M. psychrophilum* (L) grow slowly but abundantly on the semisolid agar media containing the water soluble palmitic acid or palmltates at +10°C, but not at 22°C nor at 32°C. Growth was visible as a creamy smooth, off white opaque growth on the agar surface. The bacterial mass increased slowly in size during 10 to 50 days of incubation. Round colonies turned light yellow with aging of the cultures. Bacilli were strongly acid fast, arranged in small to large clumps. At 32°C most of the cells became granulated or beaded after 20 to 30 days of incubation. The growth is seemingly maximal at 50 to 100 days of incubation. At that time cultures were regularly transferred into fresh homologous agar media. Heavy inoculum was used for transfer to fresh media. No changes in growth pattern were observed when cultures were transferred to the fresh semisolid media. None of the subcultures grew in 7H9, Loewenstein or Dubos media at 32°C.

Counting of bacilli was not feasible in semisolid media because of difficulties of declumping with chloroform. Percentage of solidly stained cells in the primary cultures and subcultures were therefore registered as an indication of the quality of cells during growth visible to the naked eye on the surface of the media. Results are shown in Table 3. The percentage of solidly stained acid fast rods is extremely low - 14% - in suspensions of leprosy bacilli, freshly isolated from a human leproma; H4, considerably higher in the armadillo derived suspensions - A6 and A7 and even higher in the inoculum obtained from Nu mice foot pads (M3). As cells multiplied in the liquid and agar primary culture, the percentage of solidly stained cells reached relatively high levels - up to 80-91% in the Nu mice and armadillo derived cultures, most likely because of cell divisions of the solidly stained bacilli.

A high percentage of solidly stained cells were counted in all the subcultures, whether of

TABLE 3 - Solidly stained cells (%) of *Mycobacterium psychrophilum* (L) isolated from human (H4), mouse foot pad (M3) and armadillo (A6, A7) lepromata. The primary cultures and subcultures were grown at 10°C in liquid and solid media.

Culture Medium	Solidly Stained Cells, Incubation at +10°C, Time in Weeks					
	Primary Culture			Subculture		
	0	4	8	0	4	8
H4 Liquid	14	28	69	75	82	86
H4 Solid	14	37	42	79	88	92
M3 Liquid	36	52	80	86	89	86
M3 Solid	36	59	84	80	92	88
A6 Liquid	24	47	69	67	88	80
A6 Solid	24	52	77	74	90	94
A7 Liquid	42	62	82	79	86	90
A7 Solid	42	70	91	88	89	89

human, mice or armadillo origin. These results are in agreement with the well known observations that most of the host grown *M. leprae* cells are fragmented, nonsolid, probably dead.

The LDM (avium-intracellulare, scrofulaceum and phiei) grow abundantly at 22 and 32°C in the 7H9, Loewenstein and Dubos media, but not on the semisolid and liquid palmitate media. None of these strains grow in any of these media at +10°C. Authentic *M. /epra*efreshly isolated from human armadillos orfoot pads of Nu mice did not grow on 7H9 media at +4, +10 or +32°C respectively. Similarly the leprosy derived

M. psychrophilum (L) did not grow in 7H9 media at any of the incubation temperatures.

Growth was not estimated in media without the palmitates added, but experience left no doubt that growth was significantly slower in the primary cultures as well as in the subcultures in the absence of palmitates.

Due to the unusual nature of the cultures, identification presented unsurmountable difficulties because test batteries are standardized for mesophilic mycobacteria. Cooperation of *extra muros* investigators was requested to solve this problem.

Investigators A and B tested armadillo and Nu mice derived *M. psychrophilum* (L) for presence of phenolic-glycolipid-1 (PGL-1) and reported strongly positive results in the 4th to 7th subcultures. Results will be reported lateras available in subcultures >10. Polymerase chain reaction tests were not done.

M7 and M3 fourth subcultures were injected into foot pads of mice and showed multiplication comparable to authentic *M. leprae* cells. Dr. M. Ishaque (IAF, University of Quebec) injected M3-13th subculture into Nu mice foot pads and will report results separately.

Dr. Eleanor Storrs (Medical Research Institute, Florida Institute of Technology) injected

A4 and M3, 13th subcultures into armadillos and will report results as available.

Dr. Enzo Melchior, Jr., Ribeirao Preto - SP - Brazil, kindly tested M3 - 6th subculture and

A5 - 7th subculture for Mitsuda type skin reactions in I, B, T and LL volunteer patients. Dr. Enzo Melchior found that the heat killed suspensions (10^7/0.1 ml) gave negative late reactions in all LL cases (as in the case of the human lepromin). In I, B, and T cases the late reaction was similar to that obtained with human lepromin.

Results are presented with Dr. Enzo Melchior's kind permission in Tables 4 and 5.

Table 6 shows some characteristics of *M. leprae* compared to *M. psychrophilum* (L) and leprosy derived mycobacteria (MAIS).

TABLE 4 - Late skin reactions (Mitsuda) in the skin of human volunteers with indeterminate (I), borderline (B), tuberculoid (T) or lepromatous leprosy (L) injected intradermally with authentic human lepromin (H: LEPR) and heat killed suspension of *Mycobacterium psychrophilum* (L), (MPL-H). Both antigens contained 10^7 acid fast cells in 0.1 ml physiological saline solution. MPL-H antigen was prepared from the 6th subculture of *M. psychrophilum* (L) (H-3) isolated from a human leproma.

Patient	Case	Skin reaction to H-LEPR	MPL-H
N.S.	I	4x4	6x6
D.C.M.	I	-	-
O.L.F.	I	-	-
V.A.T.	B	-	-
I.R.S.	T	15x15	13x15
M.I.G.S.	T	12x12	10x9
C.R.R.	T	6x11	4x6
A.M.G.F.	T	8x8	7x8
A.F.S.	T	5x6	10x9

Patient	Case	Skin reaction to H-LEPR MPL-H
N.M.B.	LL	
R.T.	LL	
A.J.	LL	
S.O. de S.	LL	
P.C.G.	LL	
M.A.G.	LL	
D.B.	LL	
J.M.T.	LL	

TABLE 5 - Late skin reaction (Mitsuda) in the skin of human volunteers with indeterminate (I), borderline (B), tuberculoid (T) or lepromatous leprosy (L) injected intradermally with authentic human lepromin (H: LEPR) and heat killed suspension of *Mycobacterium psychrophilum* (L), (MPL-A). Both antigens contained 107 acid fast cells in 0.1 ml physiological saline solution. MPL-A antigen was prepared from the 7th subculture of *M. psychrophilum* (L) (A-5) isolated from an armadillo leproma.

Patient	Case	Skin reaction to H-LEPR	MPL-H	Patient	Case	Skin reaction to H-LEPR	MPL-H
E. de O.	I	6x8	5x8	H.A.	LL		-
W.P.	I	6x5	8x8	J.M.T.	LL	-	-
J.C.S.O.	I	5x6	5x6	J.O.B.	LL	-	-
J.A.M.	I	8x7	6x6	D.R.B.	LL		-
D.F.T.	B		-	A.R.G.	LL		-
A.C.S.	BT	Eryth. 3	Eryth. 6	R.A.S.	LL		-
P. Ap. V.	T		2x2	A.W.	LL		-
M.S.	T	4x6	6x6	J.A.M.	LL	-	-
I.B. de S.F.	T		8x5				
C.A.S.	T		8x5				

TABLE 6 - Characteristics of *Mycobacterium /eprae,* compared to Mycobacterium psychrophilum (L) and leprosy derived mycobacteria (MAIS)

Characteristics	Leprosy derived Mycobacteria (MAIS)	Authentic Myco-bacterium Leprae	Mycobacterium Psychrophilum (L)
1. Regularly isolated from M. leprae infected tissues			
2. Cultures and subcultures only in special media and conditions			
3. Growth in 7H9, Dubos Loewenstein or Ogawa media	-	-	-
4. Acid fastness	-	-	-
5. Phenolic glycolipid-1	-	-	-
6. Polymerase-chain reaction	-	-	
7. Late skin reaction in lepramatous leprosy patients			
8. Late skin reaction in tuberculoid leprosy patients	-	-	
9. Multiplication in mouse foot pad	-	-	
10. Leprosy in the armadillo	-	-	

4. DISCUSSION

The presented experiments were initiated and conducted according to a previously formulated working theory as follows: LDM are an integral part of the pathology of leprosy as etiological cofactors 12. With a complete cytochrome systems, Fe is an essential element for growth. M. leprae, however, seems to be deficient in the Fe transport system". This deficiency might be supplemented in vivo by LDM, thus providing Fe transport molecules in the host. M. leprae was therefore proposed bythis authorto be a "microbe dependent microbe" "02. In vitroeithermycobactin-exochelon, or preferably an artificial Fe transport complex, is desirable in a chemically well defined medium 12'15. 17. All components of the medium - palmitate and the Fe-transport complex included - must be biologically available as water soluble complexes, or fine molecular inclusion bodies.

Special attention was given to the observations of Binford 4 that "M. leprae grow best in the cooler tissues of humans". A similar association between damage from leprosy and temperature was reported by Brand 6. These findings raised the question as to whether temperature cooler than the coolest parts of a human or an animal body is optimal for growth of M. leprae. How cool is cool enough for M. leprae to grow faster (or at all) in vitro as the well documented slow growth in vivo? Does M. leprae not spare the skin fossae, intergluteal regions and other warmer areas, as shown by Brand 6. In cool parts of the body the lesions are heavily parasitized by M. leprae, but the proliferation of leprosy bacilli is limited to microscopic lesions in warmer parts of the body 4' s. The thermal difference between normal body temperature and the cool sites is only a few degrees - not more than 4 centigrade. However, this small thermal difference is sufficient to result in heavy growth at the cool site to limited growth at the warm site of the narrow thermal spectrum. These facts suggest that the growth temperature relation is optimal for multiplication at a temperature much lower than ever recorded in the living body of any of the hosts susceptible to leprosy. Exploration of possible in vitro psychrophilic characteristics of M. leprae seemed imperative.

A special, chemically well defined culture medium was necessary to grow the leprosy derived M. psychrophilum (L).

1. The medium contained an Fe-cyclodextrin complex, with known Fe transporting siderophore characteristics (17). Fe~ forms an AC-E trisubstituted complex with cyclodextrin as shown by nuclear magnetic resonance analysis of Ling et a1.17. The complex was described as a new artificial siderophore.

2. It was necessary to supplement the medium with an SH group compound, ammonium thioglycolate. It was previously shown that SH compounds are the rare substrates which M. leprae can oxidize s.

3. The water soluble palmitate was the key component of the culture media.

This compound merits special attention as a carbon and energy source. Experimental evidence is accumulating which suggests that palmitic acid might play a major role in the energy- metabolism and probably in the energy-dependent biological processes of M. leprae.

Franzblau (6) reported oxidation of palmitic acid by M. leprae in an axenic environment. Oxidation of palmitic acid resulted in increased synthesis of energy rich phosphate (ATP) and of PGL-1 7. Using manometric technics, Ishaque 1° provided direct evidence that M. leprae oxidized palmitic acid. This was a b-oxidation process via the tricarboxylic acid cycle and the electron-transport chain with 02 as the terminal electron acceptor. Cytochromes of M. leprae were reduced by palmitic acid and fully oxidized by O2. Palmitic acid was more actively oxidized than any other substrate known to be oxidized by M. leprae. Since the 16C fatty acid provides close to three times more energy than glucose, palmitic acid should be considered as a candidate energy source in axenic media for M. leprae.

Wheeler et al. 21 expressed the view that fatty acid synthetase activity of host grown M. leprae being very low, it was insufficient to provide enough fatty acids for growth. "Hence M. leprae requires an exogenous source of fatty acid." The authors propose that since in vivo grown mycobacteria scavenge lipids from host cells, a source of lipid might promote in vitro growth of M. leprae. As shown by Barclay and Wheeler3, M. leprae can

indeed hydrolyze lipids and release fatty acyl. Fatty acid acquisition from media is therefore an important step in the synthesis of lipids, necessary for multiplication. At least one of the fatty acids might have another role of primary importance: a powerful source of energy. This became evident in the experiments of Franzblau (6) and Ishaque 10, also supported bythe results of Wheeler and Ratledge 20, concerning CO2 evolution when incubating M. leprae with palmitic acid.

The above results were obtained with host grown M. leprae, thus adapted to utilize chemistry of the host cells. When proposing palmitic acid as an energy and carbon source in vitro, one must also consider that free fatty acids are scarcely available in the host /0. The slow growth of M. leprae in vivo is probably due (among other reasons) to the dependence on host constituents, where massive scavenging of host molecules precedes utilization of substrates, whether as an energy source or as building blocks for further biosynthetic processes.

Palmitic acid and its salts are Insoluble in water. It was used in biological systems as a suspension, or in liposomes. In none of these conditions is palmitic acid biologically easily Available in a solution for mycobacteria, although most microbes can scavenge any substance from a solid state.

Water soluble palmitic acid, Na palmitate or ascorbic palmitate was incorporated into the multifactorial semisolid and liquid media. This method is based on results reviewed by Szejtli [19]. Incorporation of lipophilic substrates into culture media with the help of hydrophilic molecules was first described by Bar [1,2]. His method permitted the preparation of media containing Na palmitate accessible for the inoculum as a fine dispersion. However, this is the first time that palmitic acid or palmitates have been incorporated into culture media perfectly soluble in water.

Results presented in this communication brought experimental evidence that there exists a leprosy derived species of psychrophilic mycobacteria. This is the first time that a mycobacterium has been cultivated optimally at an incubation temperature as low as 10°C compared to practically no growth at 22°C and 32°C. It did not become evident whether these cultures were identical to M. leprae. They are certainly related to the disease, since identical psychrophilic cultures were obtained from 7 out of 9 leprosy infected armadillos, 2 out of 4 M. leprae infected Nu mice foot pads and one out of two human lepromata.

SUMMARY - *Cultivation trials for Mycobacterium leprae resulted in growth of Mycobacterium psychrophilum (L). Media were inoculated with host grown Mycobacterium leprae cells from armadillo tissues, Nu mice foot pads or human lepromata. Cultures were obtained in liquid and on semisolid multifactoria I media containing water soluble palmitic acid or its salts. Ammonium thioglycolate and Na palmitate served as carbon and energy sources. The watersoluble palmitic acid remained in perfect solution following sterilization in the autoclave, thus easily accessible to the cells. The cyclodextrin-Fe complex served asa siderophore to grow the obtained leprosy derived psychrophilic cells. The leprosy derived cultures and subcultures grew optimally at 4-10°C, but deteriorated rapidly at +32°C in the multifactorial media. No growth occurred in 7H9 media. Cultures were not identified for classification.*

Note: *Water soluble palmitic acid culture media are available from this author free of charge for qualified investigators.*

Key Words: Bacteriology of leprosy, Mycobacterium leprae, psychrophlla, culture media, palmitic acid, palmitates, Mitsuda reaction.

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