**ATTEMPTS TO APPROACH TO THE CLARIFICATION
OF CHEMICAL COMPOSITION OF MYCO-
BACTERIUM LEPRAEMURIUM***

**I. SEPARATION OF THE BACILLI FROM THE SUB-
CUTANEOUS TISSUE OF RAT INFECTED
WITH MYC. LEPRAEMURIUM, AND SOME
PROPERTIES OF THE BACILLI**

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Because of undiscovery of pure culture method of *Myc. lepraemurium* in vitro, the bacterial cells have not been separated purely from infected tissue. Any study on the chemical composition of the bacilli, therefore, has not been reported. However, the pure isolation of the bacterial cells from infected tissue and the studies on chemical composition of the bacilli should be attempted in order to approach toward determination of their properties and discovery of the pure culture method of the bacilli.

Hanks (1) and Gray (2) reported the isolation of the bacilli from infected rat testicles employing Hogeboom, Schneider, and Palade's method (3) for isolation of mitochondria, for the studies on the metabolism of murine leprosy bacilli. Toda and Lew (4), and Dharmendra and Mukherjee (5) reported also the isolation of pure bacterial cells by means of toluol or chloroform fractionation for preparing the antigen of Mitsuda reaction (6).

The purpose of this present paper is to report the efforts toward the isolation method of *Myc. lepraemurium* from infected tissue by several fractionations employing organic solvents, and the clarification of some properties of the bacilli which are obtained by the method mentioned above, and to determine whether chemical differences exist between *Myc. lepraemurium* and other acid-fast bacilli which can cultivate in vitro or not.

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MATERIALS AND METHODS

Bacterial materials. The subcutaneous lepromas were obtained from infected rat inoculated ten to twelve months before use with the Fukuoka 1st strain of *Myc. lepraemurium* which was isolated from the wandering rats in Fukuoka, Japan, in 1938, and was maintained by serial passages subcutaneously at intervals of three or five months in white rats.

Nitrogen content. The nitrogen content of all materials was measured by the micro-Kjeldahl method.

Total phosphorus. The total phosphorus of all materials was measured by Fiske and Subbarow colorimetric determination method (7).

Determination of amino acid contained in the materials. Amino acid contents were determined qualitatively by paper chromatography. Hydrolysates of the materials were obtained by heating 5–10 mg of dried bacterial cells in 2 ml of 6N hydrochloric acid in sealed tubes at 100 C for 10 to 12 hours. The hydrochloric acid in the hydrolysates was removed by distillation at low pressure. Then, with phenol and butanol as solvent, expansion was carried out by two dimensional method. After drying, ninhydrin indicator was spread upon them to demonstrate color.

Infectivity of the materials. The infectivity of the fractionated materials was tested by observation of the leprosy changes in the subcutaneous tissue after two to three months of inoculation into white rats.

Hanks' method for separation of the bacilli. This method is described in the paper of Hanks report (1) mentioned above.

Control materials. For the sake of comparison, the human tubercle bacterial cells, H37Rv strain, and the saprophytic acid fast bacterial cells, *Myc. phlei* strain, which were grown in Sauton's medium, were also prepared by the same method as the leprosy materials.

Procedure in general. The murine leprosy lepromas were homogenized with distilled water and suspended in a ten fold dilution. The suspension was immediately frozen completely in the dry-ice ether. After one to two hour in this condition, the frozen suspension was thawed completely with running water. By repeating this procedure three or four times, the bacterial cells were separated from infected cells. This suspension was added to a large amount of the distilled water, agitated slowly for a few minutes, and allowed to stand at 4 C overnight. The supernatant fluid was separated from the sediment produced while standing, and filtered through the filter paper No. 2. Toluol or chloroform was added to

this filtered material in the rate of one third, and agitated powerfully. The mixture was centrifuged at 3000 r.p.m. for 15 minutes, and separated into three fractions composed of water-, middle-, and toluol- or chloroform- layer. The quantity of the bacilli and blue staining components in each fraction was measured by Ziehl-Neelsen's and Loeffler's or Pfeiffer's stains.

RESULTS

Separation of Myc. lepraemurium from infected tissue by fractionation of chloroform. Each layer, obtained by chloroform fractionation was turned into the same volume of the original. Each 0.01 ml of three fractions was smeared in one cm square on a slide glass, and was stained by Ziehl-Neelsen's method. Thus, quantity of the bacilli contained in each fraction was microscopically measured, and then was converted in the amount of one ml. The effect of the chloroform fractionation on the pure separation of the bacilli from infected tissue was indicated by the bacilli content in mg N per ml of the material.

The results obtained are shown in Table 1. The results demonstrate that most of the bacilli which are contained in the starting material are concentrated into the middle layer by means of chloroform fractionation, and that most of non-bacterial components are removed from the starting material. In other words, the middle layer obtained by chloroform fractionation contains approximately 100-fold concentration of bacilli per mg N compared with the starting material.

TABLE 1
*Bacterial cells and nitrogen content of three layers
obtained by chloroform fractionation**

Materials	Bact. cells/ml	mg N/ml	N removal (%)	Bact. cells/mg N	Tissue staining	
					Loeffler	Pfeiffer
Starting material	3.672×10^9	0.386		9.27×10^8	∞	∞
Up layer	1.2241×10^6	0.260	32.7	4.70×10^6	4.08×10^5	1.02×10^6
Middle layer	4.692×10^9	0.109	71.8	4.29×10^{10}	2.55×10^6	1.83×10^6
Chloroform layer	7.752×10^7	0.100	74.0	7.75×10^8	∞	∞

* The results indicate average values of those obtained in three experiments.

Separation of Myc. lepraemurium from infected tissue by fractionation of toluol. This experiment was carried out by toluol fractionation instead of chloro-

form mentioned above. The results obtained are shown in Table 2. As the experiment by chloroform fractionation, the results indicate that most of bacilli which are contained in the starting material are concentrated in the middle layer, while most of non-bacterial protein are transferred into the under layer.

TABLE 2
*Bacterial cells and nitrogen content of three layers
obtained by toluol fractionation**

Materials	Bact. cells/ml	mg N/ml	N removal (%)	Bact. cells/mg N	Tissue staining	
					Loeffler	Pfeiffer
Starting Material	3.672×10^8	0.386		9.27×10^8	∞	∞
Toluol layer	4.080×10^5	0.112	71.0	3.64×10^6	1.42×10^6	8.16×10^5
Middle layer	2.652×10^9	0.067	98.3	3.94×10^{10}	9.38×10^6	6.73×10^6
Under layer	6.446×10^7	0.190	50.8	3.39×10^8	∞	∞

* The results indicate average values of those obtained in two experiments.

Isolation of Myc. lepraemurium by Hanks' method. The results obtained are shown in Table 3. It indicates that the final fraction contains ten-fold bacilli per mg N of the original material. As Gray (2) reported it is thought that this low yield of bacilli depends upon selection of the starting material.

TABLE 3
*Isolation of Myc. lepraemurium by Hanks' method**

Materials	Bact. cells/ml	mg N/ml	N removal (%)	Bact. cells/mg N	Tissue staining	
					Loeffler	Pfeiffer
Starting Material	1.836×10^9	0.2604		7.05×10^9	∞	∞
Final	3.468×10^9	0.1428	53.7	2.42×10^{10}	3.06×10^6	1.12×10^7

* The results indicate average values of those obtained in two experiments.

Infectivity of concentrated bacilli. Infectivity of the concentrated murine leprosy bacilli obtained by chloroform fractionation and Hanks' method was tested by inoculation into white rats. Two months after inoculation of the materials, leprosy changes in the site of inoculation was examined bacteriologically and histologically. The results state that the leprosy changes in rats were observed only in the case of inoculation of the concentrated bacilli obtained by Hanks' method,

and not recognized in the case of inoculation of the bacilli obtained by chloroform fractionation.

Some chemical properties of concentrated bacilli. The concentrated bacilli which were spread on a glass dish were dried at 50 C until the material had the constant values in weight, and then the material dried was employed as the starting material. Nitrogen and phosphorus content in the material was measured by micro Kjeldahl method and Fiske and Subbarow method respectively. The results are shown in Table 4. From the results, it is clear that approximately 5 to 7 per cent of nitrogen are contained in each bacterial cell of *Myc. lepraemurium*, *Myc. tuberculosis* H37Rv, and *Myc. phlei*, and that the phosphorus content of *Myc. lepraemurium* is two times as much as that of *Myc. tuberculosis* and *Myc. phlei*.

TABLE 4
*Nitrogen and phosphorus content in purified Myc. lepraemurium, Leproma, Myc. tuberculosis, and Myc. phlei**

Materials	Nitrogen			Phosphorus		
	Dry weights of material	N content	N content (%)	Dry weights of material	P content	P content (%)
<i>Myc. Lepraemurium</i>	6.1 ^{mg}	0.347 ^{mg}	5.7	8.0 ^{mg}	52- 58 ⁷	0.65-0.72
<i>Leproma</i>	12.8	1.293	10.1	14.7	98-108	0.66-0.73
H 37 Rv	12.5	0.824	6.5	11.5	38- 42	0.33-0.36
<i>Myc. phlei</i>	9.1	0.701	7.7	5.5	24- 27	0.43-0.47

* The results indicate average values of those obtained in two experiments.

Amino acid content of the material. Amino acid assays of four materials, i.e. purified *Myc. lepraemurium*, *leproma*, *Myc. tuberculosis*, and *Myc. phlei* were carried out by paper chromatographic method. No significant differences between these materials were observed and it was confirmed that twelve amino acids were contained in them, that is, alanin, aspartic acid, glutamic acid, glycine, leucine, methionine, phenylalamine, serine, threonine, tryptophane, tyrosine, and valine.

DISCUSSION

The investigation on the chemical construction of the bacilli is one of the most necessary methods to solve the mysteries which surround the leprosy bacilli. First of all, it is necessary to study the pure separation of the bacilli from infected

tissue. In this paper, some attempts of the pure isolation of the bacilli from infected tissue were reported.

Chloroform, toluol fractionation, and Hanks' method for isolation of the bacilli from the subcutaneous leproma were employed. Chloroform and toluol fractionation are hopeful methods for this purpose, but the apparent ratio of red bacilli to blue-staining non-bacterial components are less than 90 to 10, and the infectivity of the bacilli are destroyed by these procedures. Hanks' procedure is an important method, because the infectivity of the bacilli are not only lost, but pure concentration of the bacilli may be obtained. The low yield of bacilli by Hanks' method, in this paper, would be due to the subcutaneous leproma used for the starting material. In Hanks' method, chloroform, and toluol fractionation, therefore, the selection of the best kind of starting materials should be studied for pure separation from infected tissue.

Nitrogen and phosphorus assays were carried out by using the material obtained by chloroform fractionation. The results obtained indicate that the presence of nitrogen content in the purified *Myc. lepraemurium*, leproma, *Myc. tuberculosis*, and *Myc. phlei* is the same values, but that the presence of phosphorus content in purified *Myc. lepraemurium* is two times as much as that of *Myc. tuberculosis* and *phlei*. This is very interesting and noteworthy fact, and the origin of abundant phosphorus in which the key of mysteries of leprosy bacilli might be present should be investigated.

As in amino acid analysis was recognized no difference among *Myc. lepraemurium*, leproma, *Myc. tuberculosis*, and *Myc. phlei*, it presumably can explain that the proteins contained in *Myc. lepraemurium* are as similar as those in *Myc. tuberculosis* and *phlei*. Further investigations of amino acid assays, however, have to be continued to expansive from in quality to in quantity.

SUMMARY

Some attempts of the pure separation of *Myc. lepraemurium* from infected subcutaneous tissue were reported. The results obtained indicate that chloroform, toluol fractionation and Hanks' method are hopeful methods for isolation of the bacilli. Furthermore, some chemical properties of the bacterial cells of *Myc. lepraemurium* obtained from the chloroform fractionation were investigated. *Myc. lepraemurium* contains approximately the same amount of nitrogen, and contains two times as much as the amount of phosphorus of other acid fast bacilli. The concentrated bacilli obtained by chloroform fractionation are found to possess no

infectivity in white rat. Amino acid contents of *Myc. lepraemurium* were determined as similar to those of other acid fast bacilli.

In future, the efforts toward the isolation of more purified *Myc. lepraemurium* and the studies on the chemical composition of the bacilli will be continued.

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