Studies on Lipids in Leprosy

3. Chromatographic Analysis of Lipid in Leprosy[1, 2]

Isamu Sakurai and Olaf K. Skinsnes[3]

It is well-known that the cytoplasm of lepra cells contains large amounts of lipids and fragmentary bacilli as well as innumerable undigested *Mycobacteria leprae*. These cells apparently are deficient in ability to digest mycobacterial cell walls ([39, 40]). Most histochemical studies of the stored lipids in lepra cells have indicated that there are large proportions of phospholipids and fatty acids. Other lipids such as glycolipids, cholesterols and neutral fat are identified in only small amounts or not at all by histochemical technics ([9, 14, 17, 19, 25, 36, 37, 40, 41, 42]). Additionally a great deal of wax D, a macromolecular compound of lipopolysaccharide and peptide, is believed to be contained in various strains of mycobacteria.

Although extensive biochemical analyses of the lipid compositions of mycobacteria, especially *Mycobacterium tuberculosis*, have been made by Anderson *et al.* ([1, 2]), Lederer *et al.* ([20]), and others ([8]), similar analyses of leprous tissues are rare. The lack of *in vitro* culture methods for *M. leprae* and *M. lepraemurium* has largely limited such studies to histochemical methods for lipid analyses which are not sensitive enough for certain kinds of lipids ([30, 36, 37]). The purpose of this study is to initiate qualitative and quantitative analysis of the lipid compositions of *M. lepraemurium* as well as murine and human leprous tissues by established chromatographic technics ([24]), and to relate these studies to the problems of the origin of stored lipids in lepra cells.

[1] Received for publication 9 April 1970.

[2] This work was supported in part by USPHS grant R-22 AI-08644 and, on total exhaustion of research funds, by classical scrounging.

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MATERIALS AND METHODS

Experimental animals. All animals used in this study were inbred young adult mice of both sexes of the C_3H strain.

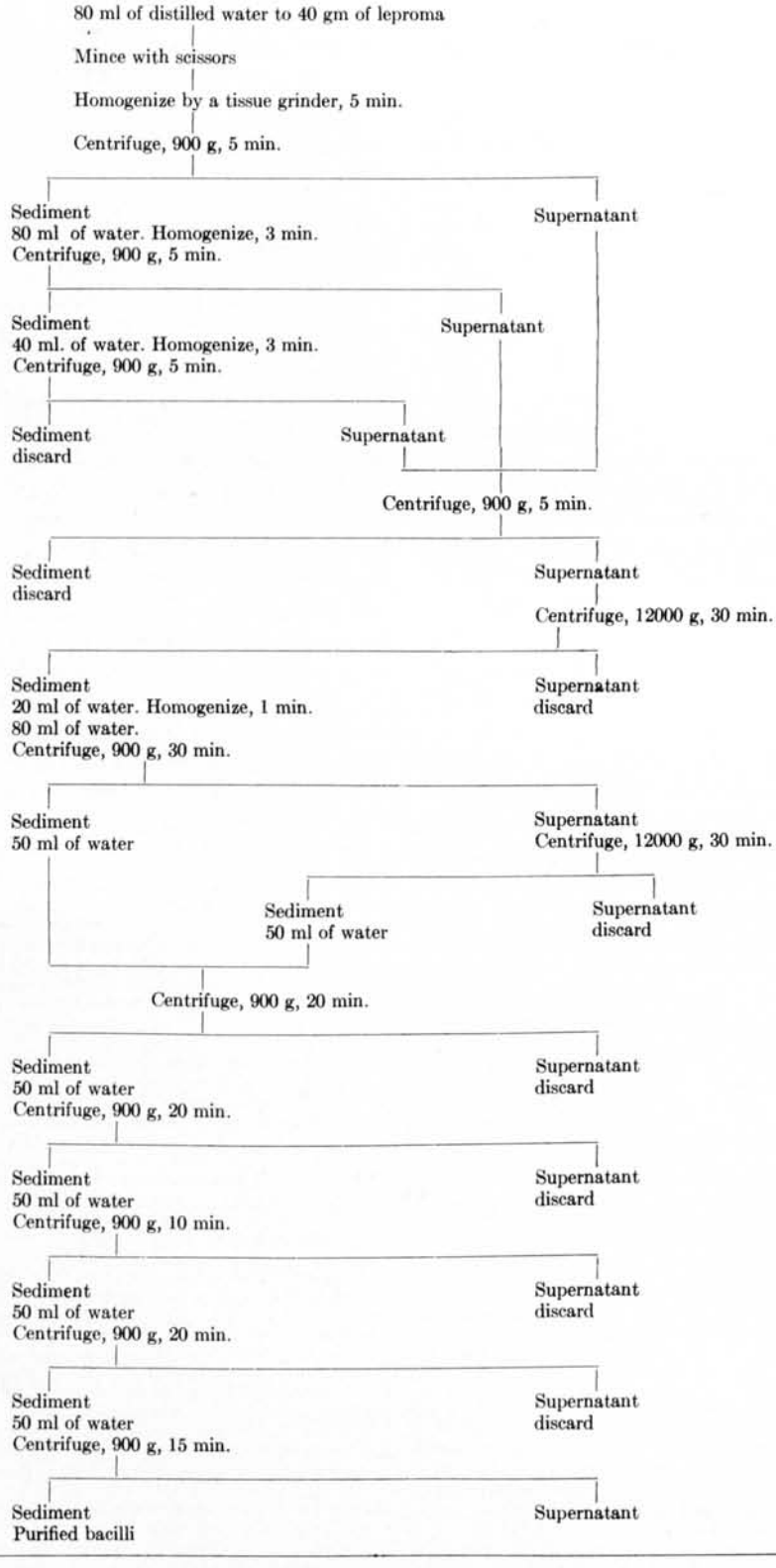
Purified *M. lepraemurium*. Purified *M. lepraemurium* suspension was obtained from lepromas of splenic and peritoneal lesions from 63 mice. These were evoked by the intraperitoneal inoculation of 0.5 ml. of crude suspension of *M. lepraemurium* (Hawaiian strain) in each mouse. Crude suspension of the bacilli for inoculation were obtained from lepromas of the infected C_3H stock mice. The lepromas were minced with sterile scissors in physiologic saline and then homogenized in a sterile Potter-Elvehjem tissue grinder for 5 minutes. The homogenates were centrifuged at 1,500 rpm for 20 minutes to remove tissue fragments before use as inoculants.

The inoculated mice were sacrificed three to eight months after infection. The spleens and peritoneal lepromas were minced in physiologic saline with scissors, and homogenized in the tissue grinder. Purification of the bacilli was performed by repeated centrifugations according to the method proposed by Nishimura *et al.* ([28]) with some modifications as shown in Table 1. The use of trypsin and ⅛ N NaOH, which are employed in Nishimura's original method, was avoided.

A total of 2.2 gm (wet weight) of purified bacilli were obtained from the lepromas of 63 infected mice. Smears of the purified bacilli were made and stained with methylene blue and Nile blue sulfate combined with Ziehl-Neelsen's stain to make certain no tissue debris remained.

Lipid extraction studies were made utilizing 1.0 gm (wet weight) of the purified

TABLE 1


80 ml of distilled water to 40 gm of leproma
Mince with scissors
Homogenize by a tissue grinder, 5 min.
Centrifuge, 900 g, 5 min.
Sediment
80 ml of water. Homogenize, 3 min.
Centrifuge, 900 g, 5 min.
Supernatant
Sediment
40 ml. of water. Homogenize, 3 min.
Centrifuge, 900 g, 5 min.
Supernatant
Sediment
discard
Supernatant
Centrifuge, 900 g, 5 min.
Sediment
discard
Supernatant
Centrifuge, 12000 g, 30 min.
Sediment
20 ml of water. Homogenize, 1 min.
80 ml of water.
Centrifuge, 900 g, 30 min.
Supernatant
discard
Sediment
50 ml of water
Supernatant
Centrifuge, 12000 g, 30 min.
Sediment
50 ml of water
Supernatant
discard
Centrifuge, 900 g, 20 min.
Sediment
50 ml of water
Centrifuge, 900 g, 20 min.
Supernatant
discard
Sediment
50 ml of water
Centrifuge, 900 g, 10 min.
Supernatant
discard
Sediment
50 ml of water
Centrifuge, 900 g, 20 min.
Supernatant
discard
Sediment
50 ml of water
Centrifuge, 900 g, 15 min.
Supernatant
discard
Sediment
Purified bacilli
Supernatant

bacilli. This mass was ground for 10 minutes with fat-free sea sand cleaned by washing with distilled water, alcohol and chloroform, while cooled in an ice bath. This method was used since Nishiura *et al.* ([29]) found by electron microscopic analyses that Braun's cell homogenizer and a simple grinding method with aluminum or glass powder yielded better mycobacterial disruption than either the French pressure cell or ultrasonic exposure.

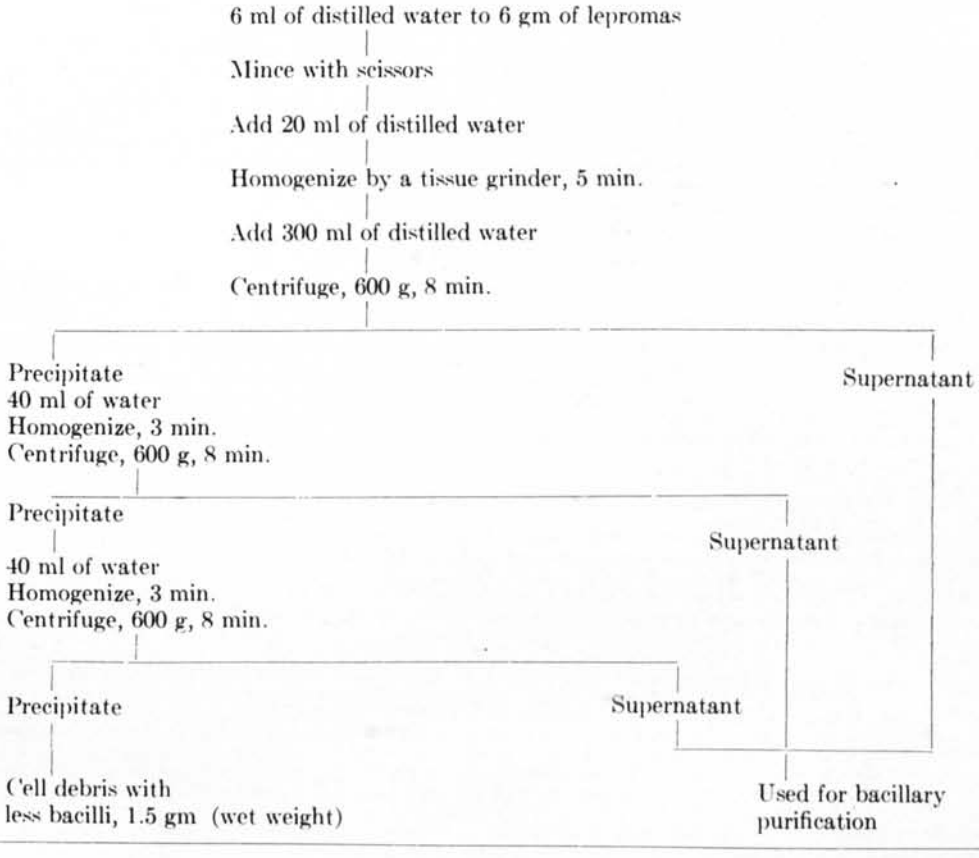
Purified *Mycobacterium phlei*. Two and a half gm (wet weight) of purified *M. phlei* were obtained by surface cultivation on broth at pH 7.4 for 5 days at 37°C. The bacilli were washed three times with distilled water, each washing being followed by centrifugation at 5,000 rpm for 20 minutes. The purified bacilli were ground with fat-free sea sand.

Murine splenic leproma. Five spleens, weighing a total of 12.0 gm (wet weight) were obtained from intraperitoneally inoculated mice five months after infection. Each spleen was greatly enlarged, and yellowish-grey, and almost completely replaced by leprous infiltration.

The spleens were minced in 12 ml of distilled water with scissors, and homogenized by a tissue grinder for 5 minutes. The homogenates were ground with fat-free sea sand in the same manner used for bacillary materials. Ten spleens from normal mice of the same age group were similarly treated as controls. They had a combined weight of 1.075 gm (wet weight).

Bacilli-free splenic lepromas. Separation of bacilli from leprous tissues was attempted by repeated low speed centrifugation to obtain murine lepra cell components without bacilli. The method employed is shown in Table 2, and was, in essence, the reverse of the method used for the collection of bacteria. One and a half grams (wet

TABLE 2



weight) of cell debris containing minute amounts of bacilli were thus obtained from 6 gm of leprous spleens from eight infected mice, which were sacrificed five months after inoculation. Smears made from the sediment cell layer and stained by Ziehl-Neelsen's method showed only a few acid-fast bacilli.

Cutaneous murine lepromas. Five and a half grams (wet weight) of cutaneous lepromas were obtained from 15 mice inoculated subcutaneously and sacrificed at eight months. None of the animals showed ulceration of the inoculated area. The mouse C_3H strain is one of the best among various strains of mice for obtaining large, nonulcerated cutaneous lepromas, as reported by Kawaguchi ([23]). Many of the lepromas had liquefactive necrosis of their centers. The lepromas were minced in 6 ml of distilled water, homogenized for 5 minutes by a tissue grinder and then ground with fat-free sand while cooled in an ice bath.

Control murine skin. Eight grams (wet weight) of skin including epidermis and subcutis, were obtained from eight normal mice of the same age group. The hairs were removed as much as possible. The materials were minced in 8 ml of distilled water and homogenized for 5 minutes.

Human leprous skin. Skin biopsies were obtained from two instances of lepromatous leprosy and one of tuberculoid leprosy from Chinese patients. The diagnoses were confirmed by histologic examination (Figs. 5, 6). Normal skin obtained from the autopsy of a patient dying of brain infarcts was used as a control. The subcutaneous tissues were removed as far as possible. The weighed specimens were kept in a freezer until they were used. Each skin specimen, including epidermis and dermis with dermal appendages and probably a little subcutaneous tissue was minced with scissors in 5 ml of distilled water and homogenized for 5 minutes in a tissue grinder.

Lipid extraction. Extraction was accomplished by the following steps, adopted from Folch *et al.* ([15]).

1. Nineteen volumes of reagent grade absolute ethyl alcohol was added for each volume of homogenized material. Solutions were agitated overnight at room temperature.
2. Solutions were filtered through fat-free Whatman glass-fiber filter-papers, which had been cleaned by washing with alcohol and chloroform, with the use of Buchner funnels and side-tubed flasks under vacuum control.
3. Filtrates were kept in a freezer.
4. Tissue residue on the filter-papers and used filter papers were re-extracted with 10 volumes of chloroform-methanol (2:1) mixture for each volume of the original homogenates for 2 hours at room temperature while agitating.
5. Solutions were re-filtered in the same manner, and filtrates were kept in a freezer.
6. Tissue residue and filter-papers were re-extracted with 10 volumes of chloroform-methanol (2:1) mixture to each volume of the original homogenates for 2 hours at room temperature while agitating.
7. Solutions were re-filtered and the filtrates were kept in a freezer.
8. Tissue residue and filter-papers were re-extracted by 10 volumes of acidified chloroform-methanol mixture (conc. hydrochloric acid 1:chloroform 124:methanol 65) for each volume of the original homogenates for 2 hours at room temperature while agitated in order to extract "firmly bound lipids."
9. Solutions were neutralized by adding 2 N NaOH.
10. Neutralized solutions were filtered.
11. Tissue residue and used filter papers were discarded.
12. All filtrates were combined.
13. Filtered solutions were evaporated completely by a Flash rotary evaporator under vacuum control.
14. Dried lipids obtained in the round bottom flasks were dissolved in chloroform by adding 20 ml chloroform 5 times to a total of 100 ml chloroform. The lipids were thus completely dissolved in chloroform.
15. The Folch method ([15]) was employed to remove nonlipid contaminants. Twenty ml of 0.73% sodium chloride were added to 100 ml of the extract and

the solutions were vigorously shaken.

16. Solutions were left for a few hours in a refrigerator, in some instances, before centrifugation or centrifuged immediately. Thereafter the chloroform and water layers were completely separated by removing the water layer through capillary suction.

17. The volume of chloroform solution remaining was usually 72 to 75 ml for each specimen.

18. The lipid containing solutions were kept in a freezer until used.

Measurement of total lipids. Total lipids of each specimen were measured by direct gravimetry. Exactly 10 ml or 20 ml of the lipid-extracted solutions were transferred by pipettes to pre-cleaned small flasks (10 ml), the weight of which was known, while the solutions were cooled in an ice bath to avoid evaporation of the solution. Chloroform was completely evaporated in a vacuum dessicator followed by a nitrogen gas flow. Each of the flasks containing completely dried total lipids were weighed on a Sartorius microbalance (capacity 100 gm; sensitivity 0.01 mg). The absolute weight of the lipids and this percentage of the unit weight of the original materials were calculated.

Separation into major lipid classes and their quantization. Separation into major lipid classes such as phospho- and glycolipid fraction, free cholesterols, free fatty acids, triglycerides, methyl esters of fatty acid and cholesterol esters was accomplished by thin-layer chromatography technic ([24,-32]). Chloroform solutions containing about 0.5 mg or 1 mg $\pm$ 0.2 mg of total lipids of the unknown and standards were applied 2 cm. above the lower edge of the TLC plates (Silica Gel H, pre-coated, 250μ thick, 20 $\times$ 20 cm., Analtech). Amounts of total lipids applied to the plates were known for each material employed.

For qualitative studies, total lipids in each sample were between 0.8 mg and 1.2 mg, but the applied chloroform solutions of samples were less than 50 μl. and contained nearly 500 μg of total lipids for quantizations.

All glassware was cleaned by alcohol and chloroform before use.

The developing solvent used was a mixture of petroleum ether-diethyl ether acetic acid (85:15:1 or 90:10:1). After the chromatograms were developed to a level 1 to 2 cm below the upper edge of the plates in a solvent-saturated tank (Analtech), they were air-dried.

Lipid migration layers were detected by spraying with 1% iodine in absolute methanol or by charring at 120°C after spraying with conc. sulfuric acid ([32]). For quantization, each layer was detected by spraying with a saturated solution of potassium bichromate in 70% sulfuric acid and then heating at 180°C for 25 minutes ([7]). Quantizations were performed by the densitometric technic detailed by Blank *et al.* ([7]), using a densitometer (Densicord Model 542, Photovolt Corp.). Densitometric quantizations of each lipid class were done by two different steps; the first was for the fractions of Rf values between 0.3 and 0.8, and the second was for the fractions of Rf values less than 0.3 and more than 0.8. According to Blank *et al.* ([7]) between Rf = 0.3 and 0.8, the peak areas are constant, but a densitometric technic for the layers having Rf values between 0.0 and 0.3, and between 0.8 and 1.0 are not constant. Thus, quantizations of phospho- and glycolipid fraction, and free and esterified cholesterols were accomplished by calculation of the peak areas of the unknown samples compared directly with those of the standards. For example, if the peak area for a 100 μg load of the standard phospholipid was 60, and the area of the unknown was 120, it was concluded that the unknown contained 200 μg of phospholipid.

For other fractions such as free fatty acids, triglycerides and methyl esters of fatty acid, the Rf values of which were between 0.3 and 0.8, the peak areas of the unknown samples were measured by a calculator (Technicon Corp., Model AAG), and percentage of the total areas from the beginning of the peak of free fatty acids to the end of the peak of fatty acid methyl esters was calculated. Absolute weights of lipids and their percentage of the total lipids could thus be obtained.

The commercial standards used were as

follows: L-α-lecithin as phospholipid; cerebroside as glycolipid; free cholesterol; oleic acid as free fatty acid; triolein as triglyceride; methyl oleate as methyl ester of fatty acid and cholesterol oleate as cholesterol ester (Sigma Chemical Co.). All analyses were performed in duplicate to avoid errors through contamination or technical errors.

Phospholipid quantization. Since phospholipids and glycolipids remain at the deposition sites on the TLC plates and can not be separated in the developing solvent used in this work, the amounts of phospholipids were determined by calculation from the amounts of phosphorus contained in the deposit sites on TLC plates. After the chromatograms were developed the lipid levels were detected by spraying with molybdate blue reagent ([11]). Only phospholipids were visualized by the blue color at the deposit sites. For quantization of phospholipids, the initial deposit area detected by spraying with 1% iodine in absolute methanol, were outlined. After the iodine evaporated, the silica gel in the outlined spots was scraped off into phosphorus-free, thick-walled test tubes. Silica gel, containing phospholipids, in the tubes was digested by adding a few small pieces of boiling chips, 1.0 ml of distilled demineralized water and 0.5 ml of 10 N sulfuric acid and then heated at 190°C for 20 minutes in a heating block. The tubes were then cooled at room temperature for 3 minutes, after which 2 drops of 30% hydrogen peroxide were added. The tubes were again heated for 15 minutes at the same temperature, cooled at room temperature and another 2 drops of 30% hydrogen peroxide were added. The tubes were heated again for 20 minutes. After cooling, 4.4 ml of distilled, demineralized water were added and the tubes heated for 20 minutes in a boiling water bath.

After digestion was completed, the amounts of phosphorus were determined by the colorimetric technic (detailed by Fiske and Subbarow ([13])), at 830 mμ wave length, by use of a spectrophotometer

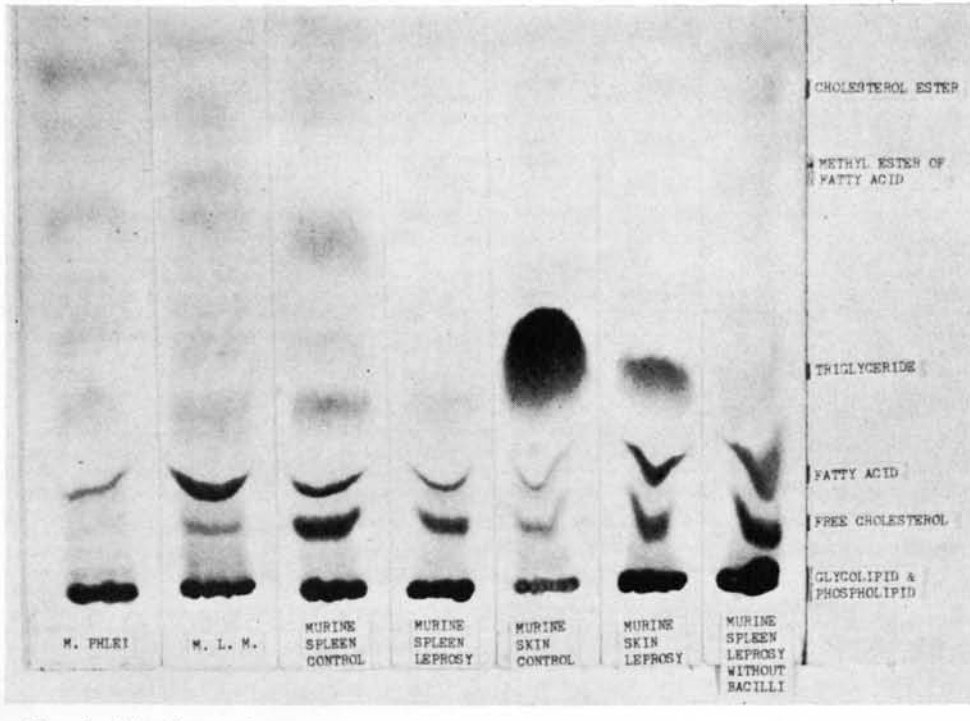


FIG. 1. Thin-layer chromatogram of lipid in murine leprosy. *Thin-layer plate:* Silica Gel H. precoated, 250 microns thick, Analtech. *Solvent:* Petroleum ether:diethyl ether: acetic acid (90:10:1). *Detection:* 100% sulfuric acid spray with charring.

(Gilford Instrument Lab., Inc. Model 2000). Color development was done by adding 0.2 ml of 5% (w/v) aqueous solution of ammonium molybdate and 0.2 ml of Fiske-Subbarow reagent (15.4% [w/v] aqueous solution of 1-amino-2-naphthol-4-sulfuric acid) and thereafter boiling for exactly 7 minutes in a water bath. The silica gel was spun down by centrifugation and the blue-colored supernatant was used for the colorimetric reading at 830 mμ wave length.

Silica gel scraped off from areas of the same size and location on TLC plates which had not been treated with any samples and which had been processed in the same manner as the sample plates, were used as blanks. All analyses were performed in duplicate. A standard solution of phosphorus was prepared from an aqueous solution of potassium phosphate.

Phospholipid quantities were calculated by multiplying the value of phosphorus by the figure 25 ([3]), since most phospholipids contain phosphorus at about 4% of their quantity.

RESULTS

Total lipid. It was not the purpose of this study to measure the total amounts of lipids in bacilli and tissues, but to analyze qualitatively and quantitatively each major fraction of the total lipids. Therefore the total lipids were not determined as dried materials, but were measured in wet weight. The results of measurements of total lipids in bacilli, and in murine and human tissues are listed in Tables 3 and 4. After complete evaporation of the organic solvents used for lipid extraction, the remaining lipids were in liquid form in the samples from normal murine and human skins, and from human tuberculoid leprosy, probably because the lipids there obtained are composed mainly of triglycerides derived from the subcutaneous fat cells. Lipids from the other specimens were in soft waxy form.

Since it is extremely difficult to remove the subcutaneous fat tissue completely from normal skin specimens of mice, the murine skin control presented a high percentage of total lipids. The homogenate of

TABLE 3. *Percentage of each lipid fraction in total lipid in murine leprosy.*

	M. phlei	*M. lepraemurium*	Spleen			Skin	
			Control	Leproma	Leproma w/o bac.	Control	Leproma
Total lipid (%) (wet weight)	1.55 waxy	7.01 waxy	3.30 waxy	7.51 waxy	3.35 waxy	28.89 liquid	9.78 waxy
Phospho- & glycolipid	49.0	39.0	25.7	37.6	31.9	4.0	27.1
Free cholesterol	2.1	7.5	20.2	27.2	20.2	1.3	20.0
Free fatty acid	31.9	28.2	24.0	14.0	34.8	2.0	7.1
Triglyceride	5.3	8.0	16.2	8.8	10.4	90.0	35.4
Methyl ester of fatty acid	1.1	4.2	9.8	4.0	0.0	0.2	0.2
Cholesterol ester	0.9	1.7	1.6	0.4	trace 0.7	0.8	0.6
Recovery	90.3	88.6	97.5	92.0	98.0	98.3	90.4
Phospholipid (%) (calculated from phosphorus)	2.1	1.5	5.3	4.9	4.1	1.3	2.4

TABLE 4. *Percentage of each lipid fraction in total lipid in human leprosy.*

	Control skin	Lepromatous		Tuberculoid
		S-2699	S-2720	S-2734
Total lipid (%) (wet weight)	1.39 liquid	3.46 waxy	5.26 waxy	10.49 liquid
Phospho- & glycolipid	7.2	26.3	31.1	7.4
Free cholesterol	3.6	8.4	10.0	1.6
Free fatty acid	2.2	30.9	24.4	3.1
Triglyceride	81.9	28.6	33.8	86.3
Methyl ester of fatty acid	2.1	1.7	0.0 trace	0.5
Cholesterol ester	1.2	3.8	0.4	0.5
Recovery	98.2	99.7	99.7	99.4
Phospholipid (%) (calculated from phosphorus)	4.2	3.5	2.8	2.0

the murine spleen control included a great deal of blood.

It is evident that murine spleen lepromas contain larger amounts of total lipids in waxy form in comparison with normal spleen, despite the normal spleen's content of blood containing serum lipids.

The tissue of human tuberculoid leprosy had more total lipid than those of the lepromatous cases employed in this study, although it is well known that lepromatous leprosy shows much lipid substance in the lesions by histochemical observations ([17,18,37]). This discrepancy may be due to more normal fat cells being included in the material from the tuberculoid specimen.

Separation of lipid classes. Separation of major lipid classes on thin-layer chromatograms indicated that the materials having total lipids in liquid form contained triglycerides in high percentage. This was observed in control murine and human skins, and in human tuberculoid leprosy.

Percentages of each lipid fractions to total lipid are shown in Tables 3 and 4.

Both *M. phlei* and *M. lepraemurium* had, as major lipid components, phospho- and glycolipid fraction and free fatty acids with several percent of free cholesterol and triglycerides. Fatty acid methyl esters and cholesterol esters were contained in small amounts in both mycobacteria. There seem to be no great differences between *M. phlei* and *M. lepraemurium*. However, when the samples of larger amounts of lipids ($1\pm$ 0.2 mg of total lipid) are applied to the TLC plates, it was found that there were two separate subfractions in the fatty acid methyl ester fraction in *M. lepraemurium*, which were not found in *M. phlei*, as shown in Figure 1. These two subfractions within the areas of fatty acid methyl esters were also observed in specimens of both splenic and cutaneous lepromas of mice (Fig. 1), and human lepromatous leprosy but they are not demonstrated in other materials.

The major lipid components of the splenic lepromas of mice consisted of phospho- and glycolipid fractions, free fatty acids and free cholesterols, despite the fact that the latter is found in murine leprosy bacilli only to the extent of several per cent of the total lipid. In addition to phospho- and glycolipid fractions and free fatty acids, murine splenic lepromas rendered essentially bacilli-free, contained more free cholesterols and triglycerides than did

purified murine leprosy bacilli.

The lipids found in cutaneous lepromas of mice were mainly phospho- and glycolipid fractions, free cholesterols and triglycerides. Free fatty acids were present only as a low percentage of the total lipid.

Free cholesterols were present in higher percentage in both splenic and cutaneous lepromas of mice than in purified *M. lepraemurium*.

The pattern of lipid fractions in the two human lepromatous cases was almost the same. The major lipids present were chiefly phospho- and glycolipid fraction, free fatty acids and triglycerides. Free cholesterols were also found in a proportion of 8% to 10% of the total lipid. The tuberculoid leprosy tissue presented a lipid pattern similar to that of skin. The demonstrated lipids were mainly triglycerides, probably derived from subcutaneous fat cells.

Phospholipid quantization. Percentages of phospholipids to total lipids are shown in Tables 3 and 4. The approximate values of glycolipids were calculated by subtraction of amounts of phospholipids from those of phospho- and glycolipid fraction.

DISCUSSION

It is well-known that mycobacterial cell walls are rich in lipids. Their compositions, especially as represented by *M. tuberculosis*, have been widely studied by many investigators ([33]). The lipid composition of *M. leprae* and *M. lepraemurium* are, however, still vague, since both have been noncultivatable on artificial culture media. Although Anderson reported studies of lipids in so-called "*Mycobacterium leprae*" ([2]), this strain has been thought in fact not to be human leprosy bacilli.

Lepra cells in human lepromatous leprosy, which evidently are deficient in ability to destroy and to dispose of the organisms, also contain large quantities of lipids ([9,-14, 17, 19, 25, 37, 40, 41, 42]), which are probably largely of bacillary origin ([17, 19,-25, 37, 40, 41, 42]). Many studies on lipids in lepra cells have been reported, but most of them are based on histochemical analyses. Accurate qualitative and quantitative analyses by biochemical technics have not been performed on leprous tissues or lepra cells.

In this study, purified *M. lepraemurium*

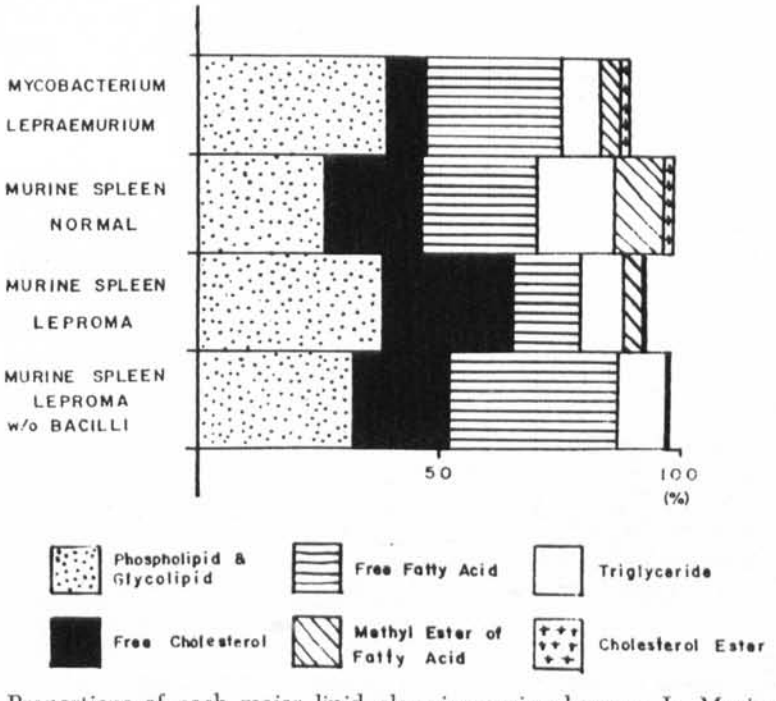


FIG. 2. Proportions of each major lipid class in murine leprosy. L. Murine splenic leproma.

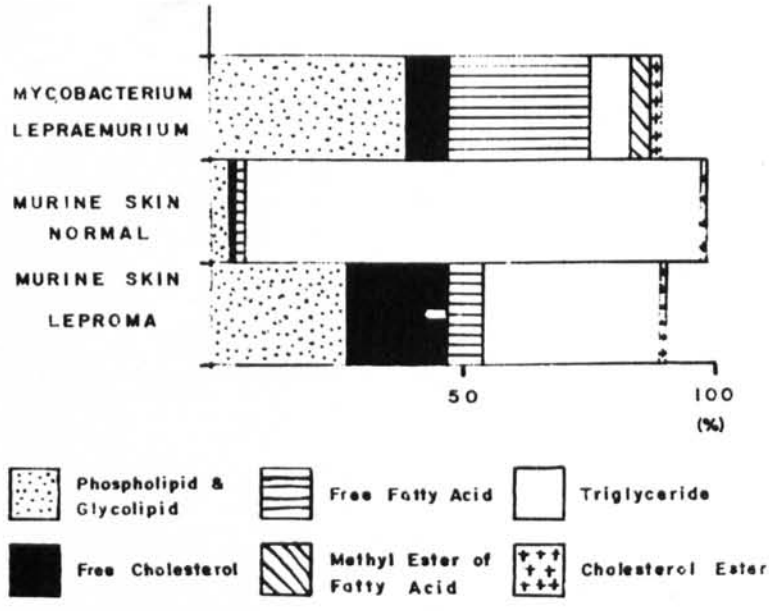


FIG. 3. Proportions of each major lipid class in murine leprosy. II. Murine cutaneous leproma.

are shown to have a total lipid content of about 7% of the total bacillary wet weight. Other investigators have reported that most mycobacteria including *M. lepraemurium* have a total lipid range of between 20% and 40% of the whole bacillary dry weight ([5, 21, 22, 26, 72]). In this study purified bacilli were measured in wet weight, since the purpose of the study was not directed to measurement of total lipids but rather on analyses of each lipid class. It is evident from this analysis that *M. lepraemurium* contains more total lipids than *M. phlei*. As indicated by Mori *et al.* ([12, 26]), however, total lipids of mycobacteria may be varied by the composition of the culture media used and by the conditions of cultivation, i.e., by surface culture or by shake culture.

M. lepraemurium lipids were found to be chiefly represented by a phospho- and glycolipid fraction and by free fatty acids. Other fractions were present only as a few per cent of the total lipids. There were no striking differences in the analyses of each lipid class between *M. lepraemurium* and *M. phlei*, except for the presence of two subfraction bands in the fatty acid methyl esters in *M. lepraemurium* on thin-layer chromatograms, which were not present in *M. phlei*. These two subfractions were found also in murine leprosy spleens and cutaneous lepromas and in human lepromatous lepromas. Analysis of the lipid content of human leprosy bacilli has not been performed because of difficulty in obtaining adequate samples, but these subfractions are thought to be of bacillary origin. It can not be assumed, however, that this peculiar phenomenon is specific to noncultivatable mycobacteria, since *M. phlei* is the only cultivatable species with which comparison was made.

Macrophages in murine leprous lesions and in human lepromatous leprosy seem to have no phagocytic dysfunction, but are deficient in ability to destroy and digest the respective bacilli ([39, 40]). Although linkages between the lipids and proteins of the bacillary structures were broken by the extraction procedures, the phospho- and glycolipid fraction separated by thin-layer chromatography may be the most important fraction related to the deficiency in host cell digestion. The substances of which these fractions are probably a part are perhaps similar to wax D, which is a ma-

cromolecular lipopolysaccharide-peptide complex, found in most strains of mycobacteria such as tubercle bacilli. Wax D was obtained by other biochemical approaches with the use of repeated extraction procedures by various solvents as proposed by Anderson or by Asselineau and Lederer ([4, 20, 43]). In this fraction may lie acid-fast wax and the phospholipids and their fatty acid components such as phthioic acid, which stimulate the multiplication of primitive connective tissue cells and induce a proliferation of epitheliod cells ([6, 16, 34, 35]). Not only purified bacilli but also murine and human lepromatous tissues have high percentages of this fraction, whereas tuberculoid leprosy, which is known to be the resistant form in the immunologic spectrum of leprosy ([39, 40]) with adequate macrophage digestion of the bacilli, presents in its lesions a very low percentage of this fraction and otherwise contains almost the same proportions of each lipid class as those found in normal control skin.

In addition to this fraction and to free fatty acids, murine cutaneous lepromas and human lepromatous lesions contain more free cholesterol than purified murine leprosy bacilli and normal skin controls. Stored lipids in macrophages, mainly consisting of phospho- and glycolipid fraction and fatty acids, are presumably of bacillary origin, but free cholesterol may be derived by lipophanerosis ([36, 37]).

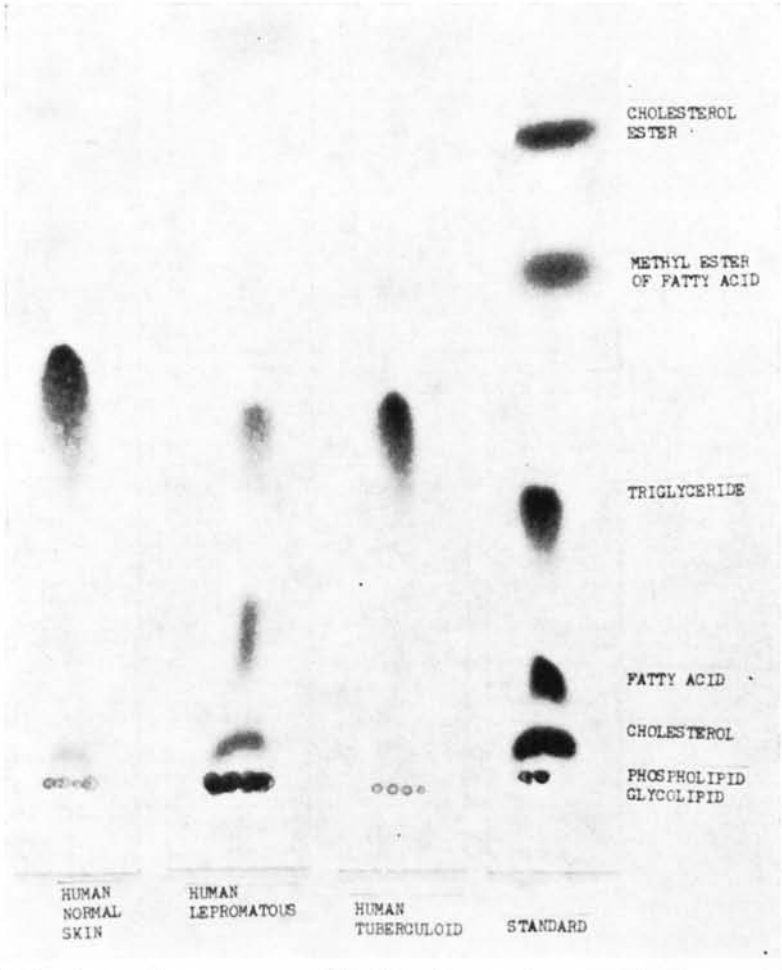


FIG. 4. Thin-layer chromatogram of lipid in human leprosy. *Thin-layer plate:* Silica Gel H. precoated, 250 microns thick, Analtech. *Solvent:* Petroleum ether:diethyl ether: acetic acid (90:10:1). *Detection:* 100% sulfuric acid spray with charring.

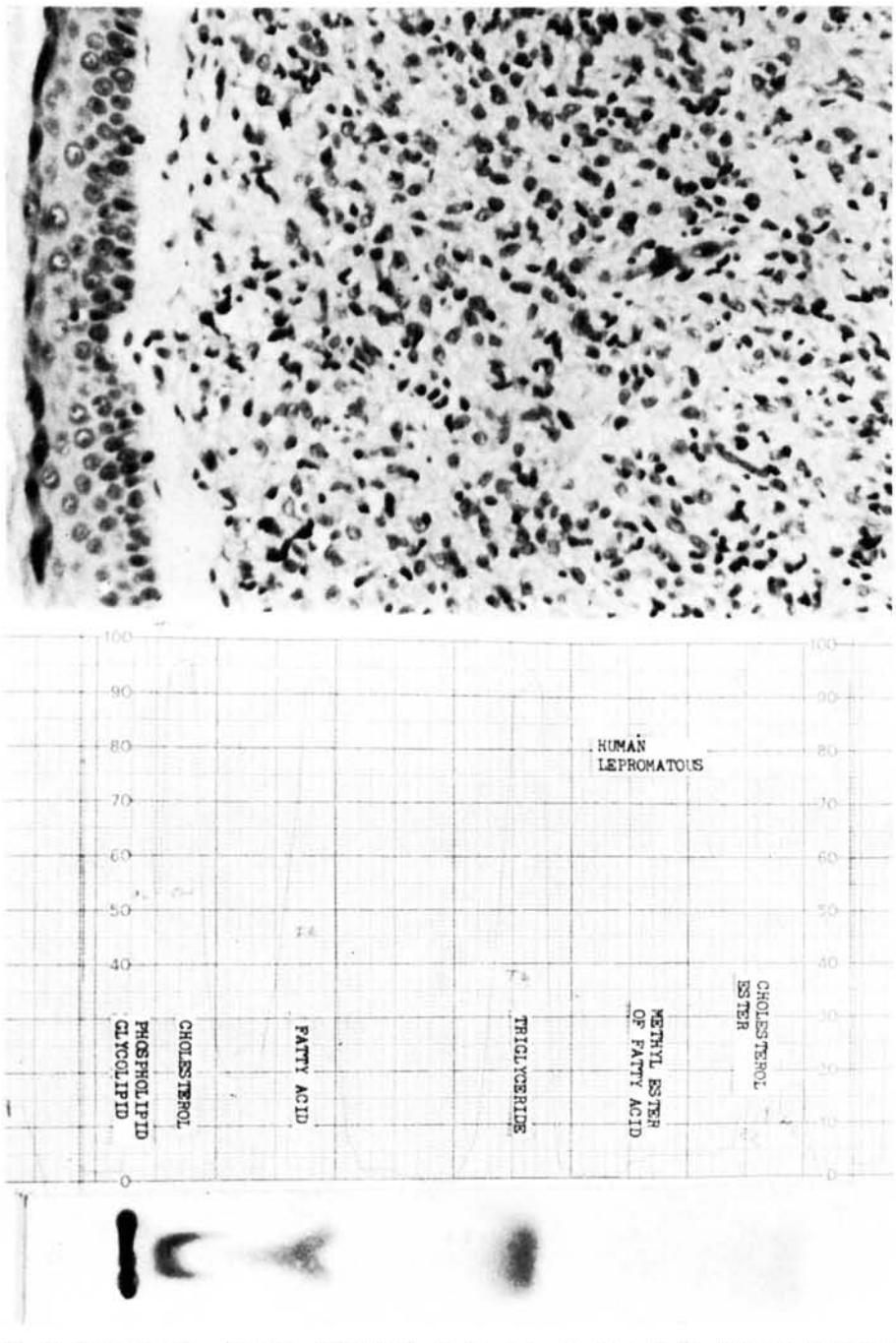


FIG. 5. Lepromatous leprosy. Histologic picture (upper) and densitiometric quantification of each lipid fractions in corresponding fresh tissues (lower).

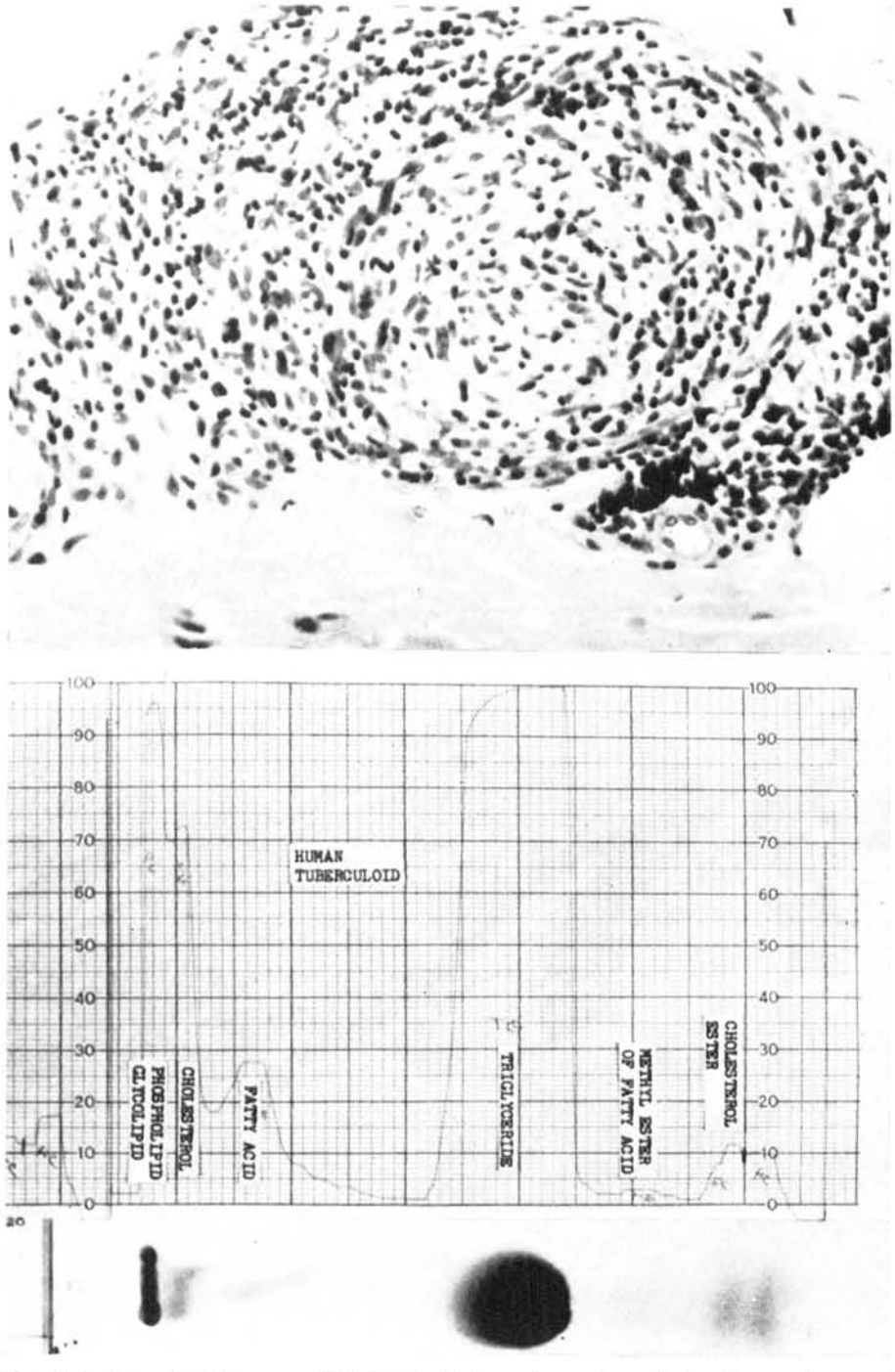


FIG. 6. Tuberculoid leprosy. Histologic picture (upper) and densitometric quantification of each lipid fractions in corresponding fresh tissues (lower).

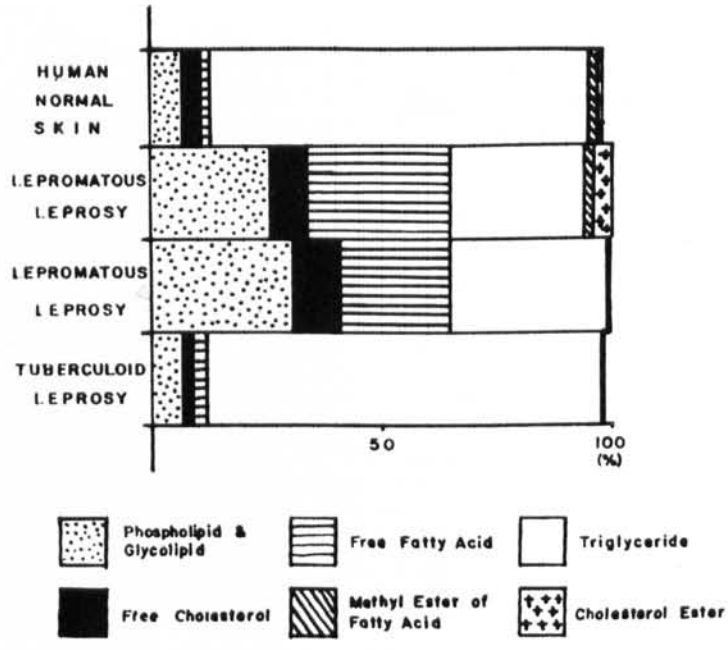


FIG. 7. Proportions of each major lipid class in human leprosy.

Most histochemical studies have indicated that the main lipid components of lepra cells are phospholipids and fatty acids ([17, 19, 25, 36, 37, 41, 42]). Dharmendra ([10]) also mentioned that phospholipids were one of the lipid components of human leprosy bacilli. The results of this study indicate, however, that the chief lipid components are phospho- and glycolipid fractions, which are probably composed largely of glycolipids, and free fatty acids, since glycolipids may be roughly estimated by subtraction of amounts of phospholipids from the total amounts of phospho- and glycolipid fractions (Tables 3 & 4). Purified murine leprosy bacilli contained phospholipids only to the extent of 1.5 per cent of the total lipid. Mori reported that phospholipids were present in a concentration of about 4% ([26]). Other investigators also have noted that phospholipids are contained only as a small portion of the total lipid in mycobacteria ([5, 43]). Mycobacterial phosphorus is, however, present in organic combinations, partly as phospholipids and partly as phosphorylated polysaccharides or glycosides ([2]). In this fraction, as shown on thin-layer chromatograms, it has not been known whether phospholipids and glycolipids are separately included or if they are present in the form of a combination as a glycophospholipid. Existence of glycolipids in lepra cells have not been proven by histochemical procedures ([17, 37, 41, 42]), probably because histochemical methods are not sensitive enough, as indicated by Okamoto *et al.* ([30]). Baker's acid hematein method and other histochemical methods for phospholipids are thought to be able to demonstrate the phospho- and glycolipid fraction shown on thin-layer chromatograms in this study.

Understanding of mycobacterial phospholipids has rapidly advanced in recent years ([31]). Ueda ([42]) reported that lecithin-like phospholipid was probably the main component of lepra cells, but lecithin has not been demonstrated biochemically in mycobacteria ([31]), and mycobacterial phospholipids mainly consist of diphosphatidyl glycerol (cardiolipin), phosphatidyl ethanolamine (cephalin), and inositol phosphatides ([31]).

As linkages between lipids and proteins were broken by the extraction procedures, electrophoretic analysis of lipoproteins was attempted in this study on aqueous homogenates of the ground murine leprosy bacilli and murine splenic lepromas. However, substances stainable with oil red O remained at the site of deposition even after electrophoresis. Existence of low density, very low density and high density lipoproteins have not been proven in these materials ([38]).

SUMMARY

1. Qualitative and quantitative analyses of lipids were performed on *Mycobacterium lepraemurium, Mycobacterium phlei*, murine leprous tissues and human leprosy lesions by a thin-layer chromatography technic.

2. The total lipids extracted were in liquid form when obtained from normal murine and human skin controls, and human tuberculoid leprosy, since they have a high percentage of triglycerides. Other materials such as both mycobacteria, and murine and human lepromas presented total lipid extracts as soft waxy forms.

3. Both mycobacteria contain large proportions of free fatty acids, and phospho- and glycolipid fractions.

4. *M. lepraemurium*, as well as murine and human lepromas contained all major lipid fractions, despite the fact that some fractions can not be identified by histochemical technics. This suggests that histochemical methods are not sensitive enough.

5. Two peculiar subfractions in fatty acid methyl esters were identified on TLC plates in the materials from *M. lepraemurium*, murine leprous tissues and human lepromatous leprosy. These subfractions, which were not present in other materials studied, presumably originated from bacillary components.

6. Murine leprosy spleen contains more total lipids than control spleens.

7. Murine leprosy cutaneous lesions contain considerably more free cholesterol than normal control skin, despite the fact that murine leprosy bacilli have only small amounts of free cholesterol. It seems reasonable to conclude that free cholesterol in lepromas presumably originates by lipophanerosis of tissues.

8. The pattern of lipid separation was almost the same in normal skin and human tuberculoid leprosy skin lesions. Both had much triglycerides of fat cell origin, and showed only small amounts of the other fractions found in lepromatous tissues.

9. Lepromatous leprosy skin contained more phospho- and glycolipids, free cholesterol and free fatty acid than normal skin control. The phospho- and glycolipid fractions and free fatty acid are probably of bacillary origin, and the cholesterol presumably originated by lipophanerosis of tissues.

Acknowledgments. The authors wish to thank Dr. Y. Hokama, Professor of Pathology, University of Hawaii School of Medicine, for his kind guidance in the densitometric analysis of thin-layer chromatograms.

RESUMEN

1. Se hicieron análisis cualitativos y cuantitativos de lípidos en *Mycobacterium lepraemurium, Mycobacterium phlei, tejidos* de lepra murina y lesiones de lepra humana por medio de una técnica de cromatografía en capa fina.

2. Los lípidos totales extraídos estaban en forma líquida cuando se obtenían de controles de piel normal murina y humana y de lepra tuberculoide humana, ya que tienen un alto porcentaje de triglicéridos. Otros materiales, tales como ambas micobacterias y los lepromas humanos y murinos, presentaron extractos totales de lípidos en forma de cera blanda.

3. Ambas micobacterias contenían grandes proporciones de ácidos grasos libres y fracciones de fosfo- y glíco-lípidos.

4. El *M. lepraemurium*, los mismo que los lepromas murinos y humanos, contenía todas las fracciones lipídicas principales, a pesar de que algunas fracciones no pueden ser identificadas por medio de técnicas histoquímicas. Esto sugiere que los métodos histoquímicos no son lo suficientemente sensibles.

5. Se identificaron dos fracciones especiales en los ésteres metílicos de ácidos grasos, en placas TLC, en los materiales obtenidos de *M. lepraemurium* tejidos de lepra murina y lepra lepromatosa humana. Estas subfracciones, que no estaban presentes en los otros materiales estudiados, presumiblemente se originaban de componente bacilares.

6. Los bazos de lepra murina contenían más lípidos totales que los bazos controles.

7. Las lesiones cutáneas de lepra murina contienen una cantidad considerablemente mayor de colesterol libre que la piel control normal, a pesar de que los bacilos de lepra murina tienen sólo pequeñas cantidades de colesterol libre. Parece razonable concluir que el colesterol libre en los lepromas se origina presumiblemente por la lipofanerosis de los tejidos.

8. El patrón de separación de los lípidos fué casi el mismo en piel normal y en lesiones dérmicas de lepra tuberculoide humana. Ambas tenían gran cantidad de triglicéridos originados de células grasas y mostraban sólo pequeñas cantidades de las otras fracciones que se encontraron en el tejido lepromatoso.

9. La piel con lepra lepromatoso contenía más fosfo- y glico-lípidos, colesterol libre y ácidos grasos libras que la piel control normal. Las fracciones fosfo- y glico-lipídicas y los ácidos grasos libres eran probablemente de origen bacilar y el colesterol se originaba presumiblemente de la lipofanerosis de los te jidos.

RÉSUMÉ

1. On a procédé à des analyses qualitatives et quantitatives des lipides, par une technique de chromatographie en couches minces, chez *Mycobacterium lepraemurium, Mycobacterium phlei,* dans les tissus de lèpre murine, et dans des prélèvements obtenus à partir de lésions humaines.

2. Les lipides totaux qui ont été extraits étaient sous forme liquide lorsq'ils avaient été obtenus dans des échantillons témoins provenant de peau normale de rats ou de peau normale humaine, de même que dans la lèpre humaine tuberculoide. Ils présentaient en effet une proportion élevée de triglycérides. Les autres prélèvements étudiés, tels les deux espèces de mycobactéries et les lépromes murins et humains, livraient des extraits lipidiques totaux qui se présentaient sous une forme de cire molle.

3. Les deux espèces de mycobactéries étudiées contenaient des proportions élevées d'acides gras libres, ainsi que des fractions phospholipidiques et glycolipidiques.

4. *M. lepraemurium,* de même que les lépromes murins et humains, contenaient toutes les fractions lipidiques principales, malgré le fait que certaines de ces fractions ne pouvaient être identifiées par des techniques histochimiques. Ceci suggère que les méthodes histochimiques utilisées ne sont pas suffisamment sensibles.

5. Deux sous-fractions particulières ont été identifiées dans les esters méthyliques d'acide gras, en étudiant les tranches de chromatographie en couches minces, dans le matériel obtenu à partir de *M. lepraemurium,* de tissus lépreux murins, et de lèpre lépromateuse humaine. Ces sous-fractions, qui étaient absentes dans les autres prélèvements étudiés, provenaient vraisemblablement de constituants bacillaires.

6. La rate des rats atteints de lèpre murine contient plus de lipides totaux que celle des rats témoins.

7. Les lésions cutanées obtenues à partir de lèpre murine, contiennent considérablement plus de cholestérol libre que la peau normale témoin, et ceci malgré le fait que le bacille de la lèpre murine ne livre que des quantités faibles de cholestérol libre. Il semble dès lors raisonnable de conclure que le cholestérol libre dans les lépromes provient vraisemblablement de la lipophanérose des tissus.

8. Le profil de la séparation lipidique était presque identique dans la peau normale et dans les lésions cutanées de la lèpre tuberculoide humaine. L'un et l'autre matériel présentaient une quantité importante de trigylcérides d'origine graisseuse cellulaire, et ne révelaient que des quantités faibles des autres fractions observées dans les tissus lépromateux.

9. La peau de lèpre lépromateuse contenait plus de phospholipides, de glygolipides, de cholestérol libre et d'acide gras libre, que la peau témoin normale. Les fractions phospholipidiques et glycolipidiques, de même que l'acide gras libre, étaient probablement d-origine bacillaire. Par contre, le cholestérol provenait probablement de la lipophanérose des tissus.

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