Comparative Study of *in vivo* and *in vitro* Grown *Mycobacterium tuberculosis*. III. Lipid Composition.* (29919)

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The classical studies on the lipid chemistry of the tubercle bacillus begun in 1927 by Anderson(1) and his colleagues set the pattern for extensive investigations conducted through the years(2). However, in a later paper(3), Anderson *et al* reported that they were unable to detect in human tuberculous lung tissue several chemical components (*e.g.*, phthiocol, tuberculostearic and phthioic acids), that had been established as characteristic of tubercle bacilli (virulent strain H37Rv) grown in culture medium. They considered that these negative results may have been due to a dilution effect by the excessive proportion of tissue present. However, they also raised the question as to whether tubercle bacilli growing in living tissue produce the same characteristic chemical compounds that are found in the case of *in vitro* culture.

Segal and Bloch(4,5) found that tubercle bacilli, separated by differential centrifugation from tuberculous mouse lungs, exhibited significant metabolic, pathogenic and immunogenic differences from *in vitro* cultured bacilli of the same virulent strain, H37Rv. These findings have been extended by elaboration of the biochemical and immunological differences between the *in vitro* and *in vivo* grown organisms(6,7). It is to be expected that significant chemical differences also exist, particularly in the light of Anderson's report (3). Evidence for the existence of such chemical differences as revealed by stain reactions is being published separately(8). The present report presents evidence for quantitative differences in lipid composition of *in vivo* and *in vitro* grown *M. tuberculosis*, as well as preliminary evidence for possible qualitative differences on the basis of gas chromatographic analysis of ether-alcohol extracts.

Methods. Growth. The *in vitro* tubercle bacilli, strain H37Rv, were grown on the surface of Kirchner's synthetic liquid medium, containing 2% glycerol. The separation by differential centrifugation of *in vivo* grown tubercle bacilli (LRv†) from tuberculous mouse (strain CF1) lung homogenates was carried out according to the method previously described(4). It has been found that there is no significant difference, with regard to biochemical, pathogenic and immunogenic characteristics, in the LRv separated after one mouse passage and the LRv separated after 25 serial mouse passages. By the criteria previously reported, plus the additional biochemical criterion of succinoxidase activity(9), tissue contamination of LRv is estimated to be less than 5%, which value is within the range of the error of analysis.

Solvent fractionation. The classical method of solvent extraction of mycobacterial lipids as originally outlined by Anderson was followed with several modifications. The washed bacilli were dried to constant weight under vacuum in a desiccator containing activated alumina. Exhaustive extraction was carried out by magnetic stirring in excess solvent, with renewal of fresh solvent at 2-day intervals until a negligible amount (less than 0.5%) of lipid was extracted (usually one repeat extraction was sufficient). Then the cells were subjected to the next solvent in the series: ether-alcohol (1:1), chloroform, and acidified (1% HCl) ether-alcohol (1:1). Extraction was carried out at room temperature for ether-alcohol and chloroform, but at 50°C with refluxing for acidified ether-alcohol. The solvent extracts were first evaporated at room temperature by means of a rotary flash evaporator under vacuum, and then to constant

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† LRv is used throughout this paper for the *in vivo* grown tubercle bacilli, separated by differential centrifugation from lung homogenates of tuberculous mice initially infected with *in vitro* grown strain H37Rv, and maintained thereafter by successive transfers from mouse to mouse.

TABLE I. Distribution of Lipid Composition of *in vivo* and *in vitro* Grown *M. tuberculosis.*

	Suspension in water	% extract* of original dry weight of tubercle bacilli			
		Ether-alcohol	Chloroform	Ether-alcohol + 1% HCl	Total lipid
LRv†	Hydrophilic	24.5	3.9	20.7	49.1
H37Rv	Hydrophobic	16.2	7.4	15.8	39.4

* Mean values each of 5 separate series of solvent extractions; significant difference in each case, $P < .01$.

† LRv is used for the *in vivo* grown tubercle bacilli, separated by differential centrifugation from lung homogenates of mice initially infected with *in vitro* grown strain H37Rv, and maintained thereafter by successive transfers from mouse to mouse.

weight in a desiccator under vacuum. A Sartorius semi-micro balance was used to determine all weights. Extreme care in quantitative technique was exercised because of the limited quantities of *in vivo* grown bacilli available for extraction. The initial quantities of bacilli used varied from 250 to 770 mg dry weight. The series of solvent fractionations was performed on 5 different batches each of *in vitro* and *in vivo* grown tubercle bacilli. The total lipid content is taken as the sum of the 3 solvent extracts in a series. Statistical analysis was applied to ascertain significance in the different mean values obtained for LRv and H37Rv.

Gas chromatography. Determination of the fatty acid composition of the ether-alcohol extracts was carried out by means of gas-liquid-chromatography (GLC). The dried ether-alcohol extracts were saponified with 1.0 N alcoholic KOH. After removal of neutrals and bases by extraction with ether from the diluted reaction mixture, the aqueous solution was acidified with HCl, and the fatty acids isolated by extraction several times with ether. The resulting mixture of acids was esterified with methyl alcohol containing 15% by weight H_2SO_4, followed by isolation and preparation of the methyl esters for analysis by GLC. This workup procedure usually gave 4-6 mg of crude esters per individual run. All GLC was carried out with a Wilkens Aerograph Hi Fi equipped with a hydrogen flame detector, and employing a $\frac{1}{8}'' \times 6'$ stainless steel column packed with 10% XE-60 (cyanosilicone gum) on 80/100 Chromosorb W which is first treated with hexamethyldisilazane. The mixture of methyl esters was taken up in CS_2, and a portion of the resulting solution injected for analysis. The oven temperature was programmed at 85 volts from 140°-300°, and then held at 300° for the remainder of the run. After this initial chromatography run, the relative carbon content of the unknown esters was determined by another GLC of a small amount of the mixture of crude esters, into which had been introduced traces of known esters ranging from n-C_{10} to n-C_{30}. The differences in band intensities on the tracing for the GLC of the unknown esters originating from the tubercle bacilli in comparison to the band intensities of the tagged mixture provided a simple and convenient method for rapid determination of the approximate carbon content of the individual esters. The total area under all the bands for each GLC tracing was measured with a planimeter and used as the basis for determining the percentages of acids found in any of the 4 groups of carbon chain length arbitrarily set up in order to compare results for LRv and H37Rv.

Results and discussion. Solvent fractionation. The results of solvent extraction, expressed in terms of per cent lipid of original dry weight of bacilli, are presented in Table I. The values obtained for H37Rv correspond closely to those reported in the literature by numerous investigators, thus providing a reliable basis of comparison to the results obtained for LRv. In the case of each of the 3 solvent systems used, as well as for the total lipid content, a statistically significant difference was exhibited by LRv. Both the ether-alcohol extract and the acidified ether-alcohol extract (the latter generally considered to be the "bound lipid" fraction) of LRv showed considerable increase over the values for H37Rv, representing increases (expressed in per cent of the H37Rv values) of 51.2% and 31.0% respectively. On the other hand the chloroform soluble lipid con-

TABLE II. Distribution of Fatty Acids in the Ether-Alcohol Extracted Lipids of *in vivo* and *in vitro* Grown *M. tuberculosis.*

	% distribution in different ranges of length of carbon chain*					
	C_{10} to C_{17}		C_{18} to C_{20}		C_{21} to C_{30}	
Exp No.	LRv	H37Rv	LRv	H37Rv	LRv	H37Rv
1	33	31	37	36	30	33
2	37	28	35	27	28	40
3	30	33	34	32	36	35
4	34	28	32	34	32	38
5	30	31	32	30	36	38
6	30		32		38	
Mean	32.3	30.2	33.7	31.8	33.3	36.8

* The relative percentages of the 4 groups of methyl esters of the fatty acids were estimated by a comparison of the area under the bands of each group with the total area for all bands in a single GLC (gas-liquid-chromatography) analysis. Determination of the relative carbon content of the esters was made by introduction of small quantities of esters of known structure into a portion of the lipid material being analyzed.

tent of LRv was found to be slightly over one-half of the value of H37Rv. It is not possible at this point of investigation to interpret these differences in the distribution of lipid components in specific chemical and biological terms. However, the low chloroform soluble lipid content of LRv is consistent with the immunological findings(7) that LRv exhibits a lower immunizing capacity, as well as diminished capacity both to induce and elicit a delayed hypersensitivity response in rabbits and guinea pigs.

The finding that LRv contains an even greater lipid concentration, representing an increase of 24.6% over the H37Rv value, presents an interesting problem for further consideration. This result was unexpected and is seemingly paradoxical in view of the hydrophilic character of the *in vivo* bacilli, in contrast to the characteristic hydrophobic behavior of *in vitro* cultured tubercle bacilli. Several possible explanations being presently investigated are suggested: 1) high concentrations of polar lipid (*e.g.*, glycolipid, lipoprotein or phospholipid) in the surface coat of LRv would account for its hydrophilic behavior; 2) this polar lipid may be absorbed superficially from the surrounding lung environment by the bacilli whose true surface is composed of hydrophobic, neutral lipids as is H37Rv; 3) or alternatively, this hydrophilic coat, originating from infected lung tissue, may be an integral part of the bacilli, or an integral part of the host-pathogen relationship. One possibility of specific absorption of polar compounds by the *in vivo* bacilli is suggested by their deficient immunizing and sensitizing capacities, namely, by the absorption of specific circulating antibodies(7). Indirect evidence which would tend to discount hypothesis 2 is that H37Rv does not become hydrophilic when mixed with and later separated from normal and mildly tuberculous lung homogenates. Furthermore, prolonged water suspension does not result in LRv becoming hydrophobic.

Interpretation of these differences in lipid distribution and total content of H37Rv and LRv requires specific chemical analysis. That differences do exist is not surprising in view of 1) the wide range of biological characteristics wherein the 2 types have been shown to differ, and 2) the highly divergent environments involved in *in vivo* and *in vitro* culture. Lipid composition has been shown in numerous examples of bacterial species, including the mycobacteria, to be a highly variable property directly determined by nutritional conditions in the culture medium, particularly the glycerol content. However, the important question that must be further dealt with in the present study is whether the observed quantitative differences reflect any qualitative differences, and if so, what is their biological significance in relation to immunogenicity and pathogenicity (LRv is doubly virulent(5)).

Gas chromatographic analysis. In a preliminary attempt to analyze these differences, the fatty acid composition of the ether-alcohol extracts was examined by means of GLC. Table II shows the general distribution of

fatty acids in the ether-alcohol extracted lipids of LRv and H37Rv. No striking differences in overall distribution of fatty acid composition of LRv and H37Rv are apparent. There is, however, a trend shown by these results in the direction of a greater percentage of longer chain fatty acids synthesized by H37Rv, although at present there is insufficient statistical basis for drawing any definitive conclusion.

Tracings for both LRv and H37Rv show a multiplicity of bands for branched-chain and unsaturated fatty acids especially above C_{18}. Palmitic and stearic acids represent the major components of the C_{10}-C_{17} and C_{18}-C_{20} fractions respectively in both the LRv and H37Rv ether-alcohol extracts. The presence of an unsaturated C_{16} and oleic acids, both of which have already been found previously in the H37Rv lipids(10), is also suggested in some of the GLC tracings for the LRv lipids by the unsymmetrical bands for methyl palmitate and stearate. A preliminary attempt has been made to analyze for the presence of C_{27}-phthienoic acid (*trans*-2,4,6-trimethyl-2-tetracosenoic acid) in the LRv lipids since it has been isolated from H37Rv and implicated in its pathogenicity (11,12). The GLC tracings for the LRv lipids consistently show a band at the proper retention time for C_{27}-phthienoate. However, it is not possible to conclude on this evidence alone that this acid is likewise elaborated by the *in vivo* grown organism because its retention time is identical with that of normal methyl pentacosanoate(13). Attempts are currently being made to isolate the compound(s) responsible for the band at the proper retention time for n-C_{25} and/or C_{27}-phthienoate.

Summary. A comparative analysis of the lipid composition of *in vivo* and *in vitro* grown *M. tuberculosis* of the same strain H37Rv has revealed a statistically significant difference in per cent lipid content for each of 3 solvent fractionating systems, as well as in total lipid concentration. Preliminary examination, by means of gas-liquid-chromatographic (GLC) analysis, of the fatty acids isolated from the ether-alcohol extracts did not show any striking difference between the two types. There is, however, a trend indicated in the direction of a greater percentage of longer chain fatty acids present in the lipids of the *in vitro* cultured tubercle bacilli. Palmitic and stearic acids are present in large amounts in both cases, plus a complex array of both normal and branched acids ranging from C_{10} without interruption up to C_{32}. The presence of the C_{27}-phthienoic acid in the lipids of the *in vivo* organism, while indicated by GLC, cannot be demonstrated solely from this evidence.

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1. Anderson, R. J., J. Biol. Chem., 1927, v74, 525.
2. Asselineau, J., Lederer, E., in Lipide Metabolism, K. Bloch, Ed., John Wiley, New York, 1960, p337.
3. Anderson, R. J., Reeves, R. E., Creighton, M. M., Lothrop, W. C., Am. Rev. Tuberc., 1943, v48, 65.
4. Segal, W., Bloch, H., J. Bacteriol., 1956, v72, 132.
5. ———, Am. Rev. Tuberc., 1957, v75, 495.
6. Segal, W., Bact. Proc., 1962, 96.
7. ———, Proc. Soc. Exp. Biol. and Med., 1965, v118, 214.
8. ———, Am. Rev. Resp. Dis., 1964, v91, 285.
9. ———, VIII, Internat. Congr. Microb., 1962, 121.
10. Cason, J., Miller, W. T., J. Biol. Chem., 1963, v238, 883.
11. Husseini, H., Elberg, S., Am. Rev. Tuberc., 1952, v65, 655.
12. Cason, J., Allen, C. F., DeAcetis, W., Fonken, G. J., J. Biol. Chem., 1956, v220, 893.
13. Cason, J., Tavs, P., Weiss, A., Tetrahedron, 1962, v18, 437.

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