Physiologic Implications of Hydrocarbons and Lipids in Mycobacteria and Related Forms

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For the last ten years we have been concerned with enzymatic reactions involved in the utilization of aliphatic hydrocarbons by a variety of microorganisms and, while the mechanisms are not germane to the fiber of this conference, our tangential preoccupation with mycobacteria may well be of concern.

Aliphatic hydrocarbons occur widely in the living world ([1,2,3,4,5]). Alkanes, branched and normal, occur in virtually every plant wax; an elegant analysis of such plant wax hydrocarbons has furnished the basis for a "chemical taxonomy" ([6]). Aliphatic hydrocarbons occur in insects ([7]), and marine organisms ([8]), quite apart from the more familiar petroleum locale.

We have found in some interesting, if not profound, surveys of hydrocarbon utilizing ability that it is quite improper to talk about "hydrocarbon organisms"—there are, in fact, no such entities. The ability to utilize aliphatic hydrocarbons as a carbon and energy source is widely distributed in nature and any large, random sampling of bacteria, fungi, yeasts and actinomycetes would, at a rough guess, yield between 10 and 20 per cent of excellent hydrocarbon utilizers among the selections. The fact that microorganisms grow at the expense of hydrocarbons may be taken as evidence that they have in them enzymes we call oxygenases, a kind of system that actually inserts an oxygen molecule into the substrate being oxidized. Thus, if the experiment is carried out in an atmosphere of O^{18} the O^{18} is actually found in the oxidation product of the substrate rather than reduced to water, as is the usual fate of oxygen in respiration. During our studies we investigated cultures from laboratory collections as well as organisms isolated from hydrocarbon enrichments. We found a most interesting situation. Depending upon how they are carried out, hydrocarbon enrichments yield pseudomonads, *Moraxella* sp., or diphtheroid forms along with an occasional *Nocardia* or *Mycobacterium*. Of the organisms selected from culture collections the *Mycobacterium-Nocardia* group proved to be by all odds the best utilizers of hydro-

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TABLE 1. *Growth of* Micrococcus *strains at the expense of normal alkanes.*

Alkane	H.O. 1-N	H.O. 3	H.O. 4	S-18.2	S-12.2	S-14.1
n-heptane	0	0	0	0	0	0
n-octane	0	0	0	0	+	+
n-nonane	0	0	0	0	+	+
n-decane	0	+	+	0	+	+
n-undecane	+	+	+	0	+	+
n-dodecane	+	+	+	+	0	+
n-tridecane	+	+	+	+	0	+
n-tetradecane	+	+	+	+	0	+
n-pentadecane	+	+	+	+	0	+
n-hexadecane	+	+	+	+	0	+
n-heptadecane	+	+	+	+	0	+
n-octadecane	+	+	+	+	0	+
n-eicosane	+	+	+	+	0	+

+ = Growth.
0 = No growth.

carbon, although they had not been selected for this character originally. We have yet to learn of a mycobacterium that will not utilize for growth one or another hydrocarbon—and the tests have included a number of pathogens, or presumed pathogens, from the diagnostic laboratory.

Since we did not emphasize mycobacteria *per se* in our studies, our figures often relegate this group to seemingly secondary importance, but the points to be made are no less valid. We speak of *Nocardia* and *Mycobacterium* as related groups and the relation is one of similar metabolism with respect to hydrocarbon assimilation rather than a taxonomic unity.

To give some notion of our assimilation tests, Table 1 shows the growth patterns of a number of *Moraxella* strains, (originally named *Micrococcus*—a name we believe now to be in error), otherwise indistinguishable from each other. It is noteworthy that even in this closely knit group there are remarkable differences in the alkanes which, in defined media, would support growth. All the strains were isolated from hydrocarbon enrichments and it is striking, for example, to note that Strain S-12.2 grows at the expense of 4 hydrocarbons and no others. There is virtually no *chemical* difference between *n*-undecane, *n*-dodecane, and *n*-tridecane, for example, and in the absence of gas-liquid chromatography it would be a difficult job to separate them; yet, somehow, these organisms can tell them one from the other. We have not even a working hypothesis at the moment to help us explain why an organism will grow at the expense of octane, for example, but not heptane.

Table 2 shows the often striking difference between an organism isolated by alkane enrichment and one selected at random; the *Micrococcus (Moraxella)* being the isolate from enrichment, the *Nocardia* a

TABLE 2. *Effect of isomerization on biologic availability.*

Compound	*Micrococcus cerificans*	*Nocardia corallina*
n-hexadecane	+	+
2-methylpentadecane	+	+
3-methylpentadecane	+	+
4-methylpentadecane	+	+
5-methylpentadecane	+	+
6-methylpentadecane	+	+
7-methylpentadecane	+	+
8-methylpentadecane	+	+
3-ethyltetradecane	+	+
4-propyltridecane	0	+
6-propyltridecane	0	+
7-propyltridecane	0	+
5-butyldodecane	0	+
6-pentylundecane	0	+

+ = Growth.
0 = No growth.

random selection. Note, for example, the far greater versatility of the *Nocardia* in growing at the expense of hexadecane isomers than that of the *Micrococcus;* this versatility of *Nocardia corallina* is paralleled by numerous species of *Mycobacterium*. The latter organisms are capable of utilizing highly branched alkanes as well as normal alkanes for growth unlike the "hydrocarbon organism," which will not grow at the expense of numerous isohexadecane molecules ([9]). Another way in which the same point may be made is found in Table 3, where the range of hydrocarbon utilization by a number of organisms (a small number of many tested) shows that organisms isolated from alkane enrichments use far fewer hydrocarbons for growth than do the organisms not selected in that fashion.

Having established that mycobacteria, unselected for any character, are excellent hydrocarbon utilizers, we must digress here for a moment. Figure 1 shows essentially what is known of the several mechanisms of hydrocarbon oxidation, and it is presented here to establish, for the purpose of this meeting, a few points to which we shall return later. Oxygen enters the hydrocarbon molecule from gaseous oxygen (oxygenase); the oxidation is primarily at the C_1 position (as opposed to chemical oxidation where the C_2 position is the preferred oxidation locus), and a fatty acid is an early oxidation product of alkane oxidations. It follows, therefore, that far-reaching oxidation and assimilation of an aliphatic hydrocarbon is tantamount to oxidation of the corresponding monocarboxylic or dicarboxylic fatty acid. And, in addition, organisms capable of utilizing branched alkanes must be capable of oxidizing and

TABLE 3. *Bacterial hydrocarbon utilization.*

Organism	Of 30 tested hydrocarbons utilized
Pseudomonas fluorescens	11
Pseudomonas aeruginosa[a]	10
Mycobacterium paraffinicum[a]	10
Mycobacterium rhodochrous	18
Nocardia erythropolis	21
Nocardia corallina	21

[a]Isolated from hydrocarbon enrichment.

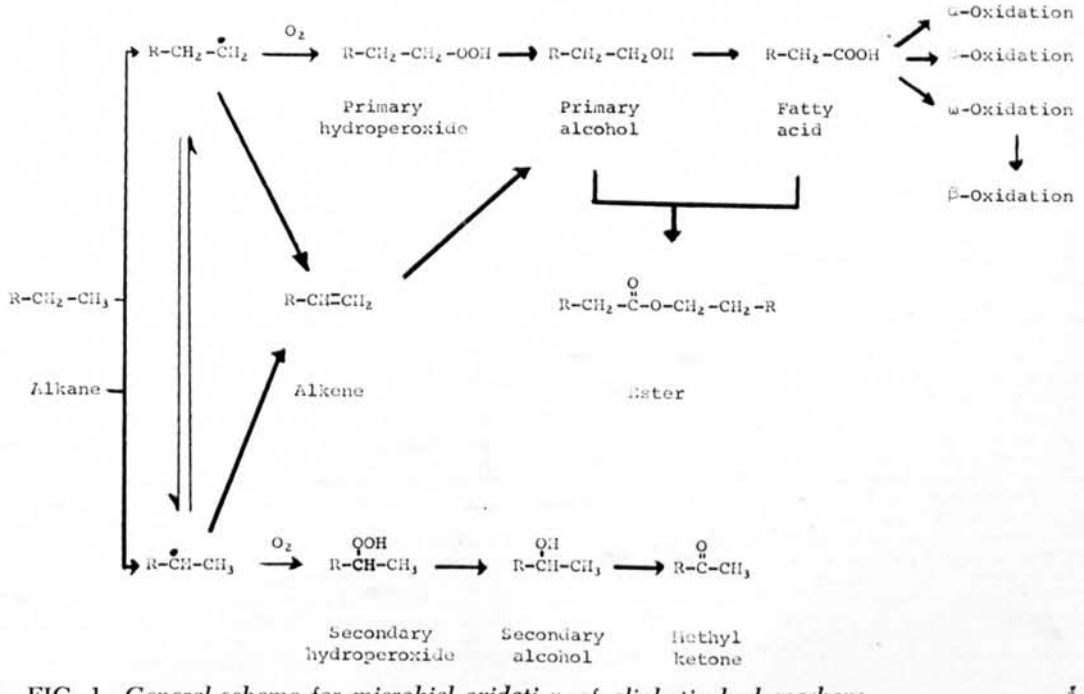

FIG. 1. *General scheme for microbial oxidati n of aliphatic hydrocarbons.*

TABLE 4. *Acetone-soluble and acetone-insoluble fractions of nocardial lipid.*[a]

Growth substrate	Cell lipid %	Acetone-soluble %	Acetone-insoluble[b] %	Composition of waxes in acetone-insoluble fraction		
				Alkyl R+ Acyl R	Alkyl R	Acyl R
Glucose	28	100	0	No wax	No wax	No wax
n-hexane	28	90	10	No wax	No wax	No wax
n-tridecane	26	87	13	No wax	No wax	No wax
n-hexadecane	48	62	38	C_{30}(14%)	C_{16}	C_{14}
				C_{32}(86%)	C_{16}	C_{16}
n-octadecane	56	61	39	C_{34}(20%)	C_{18}	C_{18}
				C_{36}(80%)	C_{18}	C_{18}

[a]In per cent of dried cell mass.
[b]Contains wax (RCOOR').

using for growth purposes the corresponding branched fatty acids.

While mycobacteria generally can utilize alkanes and fatty acids with one or more methyl branches for growth, there is a limit to the number and placement of methyl groups that can obtain and still yield an assimilable molecule. Thus, quaternary carbon atoms make a molecule difficult even for mycobacteria to oxidize. Since, however, the actual occurrence of such quaternary carbons is extraordinarily rare in nature, these cases may be dismissed as being artifacts of the chemistry laboratory. There are other obstacles to the utilization of alkanes (and fatty acids). For example, a phenyl group in the near-center of a long normal alkane chain, i.e., 9-phenyleicosane, makes for a nonoxidizable substrate; these, however, are not important to us here.

To return to the mycobacteria, it is interesting to note that thus far no mycobacterium has been isolated which specifically requires an alkane for growth. Perhaps there are such organisms, and perhaps they require *branched* alkanes (or fatty acids), which in view of their branched fatty acid compositions would not be unusual. As Dr. Goldman is fond of pointing out, mycobacteria *make* fatty acids—availability of branched naturally occurring alkanes just might make it easier for some mycobacterium to synthesize its branched fatty acid componentry. It would seem that this is an intriguing avenue of approach. For instance, the highly branched alkane, 2,6,10,14-tetramethyl pentadecane (pristane) occurs widely in nature ([10]) and we know it is readily assimilated by mycobacteria and other microorganisms. Fatty acids with similar structures are also known to occur ubiquitously ([11]). Thus it is tempting to speculate that there may indeed be mycobacterial forms that specifically require these methyl-branched materials for synthesis of methyl-branched cell components.

TABLE 5. *Fatty acid composition of the triglyceride fraction of nocardia cells grown on n-alkanes.*

n-alkane substrate	Fatty acids produced
C_{13}	C_{13}[a], C_{15}, C_{17}
C_{14}	C_{12}, C_{14}[a], C_{16}, C_{18}
C_{16}	C_{12}, C_{14}, C_{16}[a], C_{18}
C_{17}	C_{13}, C_{15}, C_{17}[a]
C_{18}	C_{12}, C_{14}, C_{16}, C_{18}[a], C_{20}
C_{19}	C_{13}, C_{15}, C_{17}, C_{19}[a], C_{21}
C_{20}	C_{14}, C_{16}, C_{18}[a], C_{20}

[a]Chain length predominating.

In a related area it might be of considerable interest to consider the change in composition that frequently accompanies changes in the nutritional history of an organism. In recent years it has become quite obvious that compositional studies of microorganisms are, for comparative purposes, useless unless the nutritional history of the culture is known. The precise

TABLE 6. *Composition of aliphatic waxes produced by a nocardia grown on C_{16}, C_{17}, C_{18}, and C_{19} n-alkanes.*

n-alkane substrate	Aliphatic waxes produced[a]	
	Alcohol component	Fatty acid component
C_{16}	C_{16}	C_{16}, 90%; C_{14}, 10%
C_{17}	C_{17}	C_{17}, 93%; C_{15}, 7%
C_{18}	C_{18}	C_{18}, 80%; C_{16}, 20%
C_{19}	C_{19}	C_{19}, 55%; C_{17}, 30%; C_{15}, 15%

[a]Specifically, for example, the n-C_{19} substrate yielded the following waxes: nonadecyl nonadecanoate, nonadecyl heptadecanoate, and nonadecyl pentadecanoate, in the percentages indicated by the per cent of fatty acid component.

metabolic role of fatty acids is conjectural, but, since they occur more or less universally, it may be assumed they have some function, and, moreover, one might expect a "basal" fatty acid composition from which point compositional analysis of cells might show quantitative deviation as a result of past nutritional circumstances. Long-chain aliphatic hydrocarbons exert a rather remarkable influence on the fatty acids of certain cells, the more so since it appears that under the stimulus of assimilating certain alkanes, qualitative changes occur in the fatty acids of *Nocardia*. Raymond and Davis ([12, 13, 14]), for example, have demonstrated considerable variation in the amount and *kind* of lipid produced by a species of *Nocardia* grown at the expense of glucose as compared to *n*-hexadecane (Table 4); total lipid in the cells was 28 per cent and 48 per cent, respectively. Cells grown on glucose were shown to have lipid composed exclusively of glycerides, whereas the qualitatively different aspect of hexadecane-grown cell lipid was the presence of considerable (38 per cent of total lipid) wax (RCOOR'), predominantly cetyl palmitate. When propane, butane, hexane, or tridecane was the carbon source, no wax was produced and the total lipid was essentially the same as in glucose-grown organisms. When, however, the chain length of the alkane was increased above 13, an interesting modification took place in the lipids of the cells—the fatty acids of both triglycerides and waxes mirrored the carbon skeleton of the substrate; e.g., growth at the expense of *n*-heptadecane produced lipid having predominantly C_{17} acids with lesser amounts of C_{15}, C_{13}, etc. (Tables 5 and 6). Hexadecane-grown cells had predominantly *even*-numbered fatty acids. These experiments clearly show that qualitative and quantitative changes can be induced in the entire lipid spectrum of a cell—what changes in immunologic, pathologic or other parameters these chemical modifications may in turn induce is moot, but it would be most interesting to learn in the genus *Mycobacterium*, which already is synonymous with unusual fatty acid metabolism.

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DISCUSSION

Dr. Long. Thank you, Dr. Kallio. Up to the present we have had little information on the role of hydrocarbons as substrates for mycobacterial growth. I will ask Dr. Byron S. Tepper, who is Microbiologist at the Johns Hopkins-Leonard Wood Memorial Leprosy Research Laboratory at the School of Hygiene and Public Health in Baltimore, to open the discussion of Dr. Kallio's paper.

Dr. Tepper. Since the topic of lipids has been introduced by Dr. Goldman and Dr. Kallio, I cannot resist the temptation to describe some of the newer information on the localization and the physiologic implications of the lipids in the mycobacteria.

As you know, most analyses of the lipid content of the mycobacteria have been based on the solvent extraction procedure devised by Anderson. With this procedure mycobacterial lipids were fractionated into several components. However, this method gave no indication of the localization of the lipids in the cell or the physiologic implications of the lipids in the cell. By comparison with other bacteria, Anderson's methods did reveal that a high content of chloroform-soluble lipids characterizes the mycobacteria. In addition, Anderson's studies showed that certain classes of lipids could be found only in the mycobacteria, i.e., tuberculostearic acid, the phthioic acid, and mycolic acid groups of lipids.

Unfortunately, very little work has been done with the lipids of the leprosy bacillus. To make this possible would require large amounts of the compounds, and we cannot cultivate the organism for such study. By studying other mycobacteria, however, we may find that some general similarities exist which might pertain to the leprosy bacillus. Some of the information we have obtained from studies of saprophytic and pathogenic mycobacteria may be applicable to the leprosy bacillus and may help us to obtain information for the cultivation of this organism.

Kotani and his associates have added to our knowledge of the localization of the mycobacterial lipids in their study of the distribution of lipids in fractions of sonically disrupted BCG bacilli. The cell preparations were extracted by the methods of Anderson, yielding seven major lipid fractions. The total lipid content of the bacilli was over 25 per cent. The cell wall fraction contained over 60 per cent of the cell's total lipids. It was of interest to note that the chloroform-soluble lipids, waxes B, C, and D were localized almost exclusively in the cell wall fraction, as were the bound lipids that can be isolated only after acid or alkaline hydrolysis of the cellular material. The acetone-soluble fats, however, were higher in the soluble and particulate fraction. Phosphatides were high in both the cell wall fraction and the particulate fraction.

Kotani's observations give us some very important observations on the location of the lipid fractions previously studied by other investigators with intact cells. High levels of phosphatide are found in the particulate fraction, a fraction obtained by high speed centrifugation containing granules of various sizes including fragments of the cytoplasmic membrane. It is probable that the cytoplasmic membrane in mycobacteria, like that in other organisms, is lipoprotein in nature. The phospholipids may be associated with the structure and enzymatic activities ascribed to this membrane and the granules in proximity to it. The association of waxes B, C, and D almost exclusively in the cell wall is also of importance, because it suggests the location of the complex lipopolysaccharides and lipopeptidopolysaccharides of the mycobacteria. The highly toxic cord factor identified as a 6,6′-dimycolate of trehalose, occurs primarily in the wax C fraction. Purified wax D of the tubercle bacillus, used commonly as an immunologic adjuvant, has been shown to contain about 50 per cent mycolic acids and 50 per cent of a nitrogenous polysaccharide. The polysaccharide contains the three sugars arabinose, galactose and mannose, linked to a peptide composed of alanine, glutamic acid and diaminopimelic acid. It is interesting to note the chemical similarity in sugars and amino acids between the peptidopolysaccharide of wax D and the cell wall of mycobacteria. The peptide moiety of wax D seems to be necessary for the adjuvant activity of this fraction. Wax D fractions derived from bovine, avian, and saprophytic strains do not contain the peptide fraction as part of their general structure and are inactive as immunologic adjuvants.

Kotani and his associates have also demonstrated a "bound wax D" as a major component of BCG cell walls. Using cell wall lytic enzymes on cell walls that had been exhaustively extracted with neutral organic solvents, they were able to isolate a cell wall component essentially identical in composition to the wax D of the human tubercle bacillus. This mycolic acid-polysaccharide-peptide isolated from the delipidated cell walls had high adjuvant activity and was definitely distinct from the usual wax D fraction isolated by solvent extraction from the same organism. This finding explains the lack of activity in the solvent extracted wax D. It also explains the adjuvant activity in bovine, avian, and saprophytic mycobacteria that had been extracted with neutral solvents, but still contained their firmly bound lipid. The data also suggest that "bound wax D" is a component of the cell wall and differs from the usual wax D, which may be located as a capsule over the cell wall proper.

From the data of Kotani, it would appear that the lipids located within the cytoplasm, either in the particles or soluble fractions,

are more readily extracted by alcohol-ether than are the more firmly bound cell wall components. It is also apparent that free lipids, which may amount to 40 per cent of the weight of the cell wall, do not seem to be integral components of the cell wall. Alcohol-ether extracts many classes of lipids from mycobacteria; in addition to the phosphatides, these include saturated straight-chain fatty acids, unsaturated straight-chain fatty acids, branched-chain fatty acids, glycerides, mycolic acids and esters of the complex fatty acids. In view of the complexity of this fraction it is hard to ascribe physiologic functions. These compounds may play an active role in the metabolism of the mycobacteria; they may be storage products or energy reserves; they may be products of detoxification mechanisms of the cells; they may be precursors of cell structures; or they may be "cementing substances" for cell components.

Considerable qualitative and quantitative variations in the content of alcohol-ether soluble lipids have been observed to depend on the mycobacterial species or strain, the composition of the medium, or the age of the culture. Our analyses of the lipid content of *Mycobacterium phlei* have shown changes in the lipid content of this species during growth on four media. Our data show the commonly observed phenomenon that glycerol produces cells with higher lipid content than cells grown on glucose. During the growth of the cultures, lipid content increases with maximal values occurring in the last part of the exponential phase of growth and during the stationary phase. These data are typical of bacterial species that synthesize lipid as a storage product in response to a nitrogen deficiency in the medium. It appears, therefore, that some of these lipids may play a similar role in the mycobacteria. The respiratory quotients of starved mycobacteria are also indicative of the utilization of lipid reserves.

The branch-chain acids of the mycobacteria accumulate toward the end of growth and not during the dividing phase. 10-D-methyloctadecanoic acid (tuberculostearic acid) is formed in *M. tuberculosis* and *M. phlei* during the last part of the exponential phase and not at all at the beginning. This observation led to the conclusion that this lipid was the end product of a detoxification mechanism. However, the discovery of a mutant of *M. tuberculosis* that requires tuberculostearic acid for growth shows that tuberculostearic acid is an essential metabolite and may play an active role in the metabolism of the tubercle bacillus.

Little is known of the degradation of the complex lipids by the mycobacteria; even less is known about the mechanism of their synthesis. Thus, it is still difficult to assess the physiologic implication of these compounds. It is an even more difficult task when one speculates that some of these lipids *may not* be necessary to the life of the mycobacteria. Virulent human and bovine strains of *M. tuberculosis* contain phthioic acids (phthienoic and mycocerosic acid group), which, so far, have not been found in the lipids of any other species of mycobacteria. The lipids of the avirulent human strain H37Ra and of the attenuated bovine strain BCG are devoid of acids of the phthienoic acid type. To confuse the issue further, Anderson and his associates were unable to isolate these same phthienoic acids or tuberculostearic acid from 2.225 kgm. of pulmonary tuberculous lesions. The lipid content of mycobacteria growing in their hosts is still unknown. Comparisons of the metabolic activities and composition of *in vitro* and *in vivo* grown mycobacteria may answer some of these perplexing questions.

Considering the complexity and uniqueness of the mycobacterial lipids, one can speculate on the possibility that the leprosy bacillus, by mutation, has difficulty in synthesizing some complex lipid that is necessary for its growth *in vitro*. Precursors of such lipids may supply necessary requirements for cultivation. On the other hand, a knowledge of the synthesis of these unique lipids may lead to the discovery of a specific chemotherapeutic agent which, by blocking the synthesis of an essential lipid, may be effective against mycobacteria in general or, more specifically, the leprosy bacillus.

Dr. Kallio has implied that a hydrocarbon, possibly an alkane, may be necessary for the growth of the leprosy bacillus. I wonder if he would speculate on the role that such compounds may play in the metabolism and cultivation of the leprosy bacillus.

Dr. Long. Dr. Tepper has asked Dr. Kallio a question that I was going to ask myself. I will rephrase it, beginning by saying that what Dr. Tepper has just said has been of a special interest because so much work has been done on the lipids of certain mycobacteria. Dr. Rudolph Anderson was furnished many pounds of cultures of different mycobacteria for his work, and—believe it or not—we gave him a pound or so of what we called "leprosy bacilli" from an old stock culture. In my office in the Leonard Wood Memorial I have two fairly good sized boxes of the Anderson lipids which I have promised Dr. Tepper. I am sure there is enough so that if some of the rest of you wish to share in them, he will be willing to give you some of the stock I am turning over to him. I was interested in his final question, hoping that we could bring out some correlation of the two presentations we have had on the lipids and the hydrocarbons. We do know pretty well the metabolism of some of the nutrients supplied to the tubercle bacillus and other mycobacteria. We know that very simple substances can be built up into the complex substances that Dr. Tepper has just described. Fortunately, since this work has started, we have been able to use the technic of radioactive tracers, i.e., use chemical compounds with a marker, to see what happened finally. I am going to ask Dr. Kallio if in turn he will discuss Dr. Tepper's paper and tell us something of the possible role of the hydrocarbons in production of the lipids of the tubercle bacillus. I would like to ask him also if in his work on this great variety of hydrocarbons he has ever had any of them marked with carbon 14 to see if he could find where they went in the course of the metabolism that he described so well.

Dr. Kallio. I would like to preface my discussions with a little background information. I believe the kinds of compositional studies that Dr. Tepper presented and that I presented at the end of my talk, are not really very meaningful in the effort to assess the role or location of a particular lipid fatty acid or other compound. For this kind of information, we have to turn to other kinds of approach, i.e., other than pure compositional studies. We must recognize that the composition of a bacterial cell varies so much that, in general, compositional analysis is worthless unless we have clear information about the immediate past biochemical history of the organism, viz., what it was grown in. I believe that most of the old compositions that have been published for bacterial cells are not worth the paper they are printed on. I think the role of fatty acids generally in biologic systems is conjectural—to be kind about the whole thing. To say that they are probably storage products, in my opinion is begging the question, for, if they were storage products, there would be no need for the bewildering array of fatty acids found in nature, particularly in the mycobacteria. There is no reason why normal fatty acids cannot be used for storage, but we find all kinds of weird branched ones. We find all sorts of acids associated in ester linkage with carbohydrates and with high molecular weight alcohols, and I suspect, for I take a very primitive view of these things, that the organism knows what it is doing and that it is making these substances for a particular purpose. It is just that we are not bright enough to recognize it. So I think, first of all, that some of these compounds are more than simply storage products. We have to find out which are necessary for the life of the organism and where they are. I believe that someone some day should make protoplasts of mycobacteria. In this way we could get valuable information as to what is in the wall and what is in the protoplast and so on. Studies of this kind are essential. As to the localization of these compounds, i.e., the portion of the cell where they are found, I suspect that Dr. Goldman has more information than anyone here present.

Now with regard to whether or not alkanes and/or fatty acids of a particular kind might be necessary as growth factors, as an absolute requirement for *Mycobacterium leprae,* let us take a very wild situation and see what happens. There is a peculiar condition known as Refsum's syndrome, a neuropathic state in which the mind remains normal and the fatty acid composition of brain material observed at autopsy is essentially normal, but the fatty acid composition of the liver, the kidney, and fat depots throughout the body shows triglycerides and cholesterol esters containing, instead of normal fatty acids, as one would expect, highly branched fatty acids. One single highly branched fatty acid, as a matter of fact, turns out to be 3, 7, 11, 15 tetramethyl-hexadecanoic acid, a 16 carbon chain with four methyl branches on it. This structure is remarkably similar to the plant alcohol phytol which is the alcohol moiety of chlorophyll. Now, let us just suppose that chlorophyll ingested in the diet has the phytol hydrolyzed away from the chlorophyll. The alcohol group of phytol has oxidized to an acid. You now have what we call phytanic acid for want of a better name. This is absorbed and incorporated into tissues in certain conditions, and if again *M. leprae* needs this branched fatty acid as an absolute requirement, you now have a selective environment for *M. leprae* based on chlorophyll metabolism. Does that answer your question, Dr. Tepper?

Dr. Long. I shall ask Dr. Goldman and then Dr. Segal to speak on these points.

Dr. Goldman. First of all, several times this morning the suggestion has been made that mycobacteria oxidize fatty acids. I would like to suggest that in the intact cell this is sheer nonsense. These mycobacteria *synthesize* fatty acids. They will oxidize fatty acids if you rupture the cell membrane and pull out the enzymes that can be made to work as an oxidizing system, but the fact remains that this bug wants to do one thing only and that is to grow and reproduce. It is not going to oxidize fatty acids unless there is rapid turnover of fatty acids in the cell, which normally does not occur. So, if you permit the cell to grow, it is going to synthesize only. It is not going to oxidize the fatty acids, because it is going to be working from glycerol.

The second point is that you can make a completely artificial fatty acid synthesizing system by taking the system analogous to the pigeon liver of the mammalian mitochondrial fatty acid synthesizing system, in which acetyl-enzyme and malonyl enzyme condense to give, ultimately, butyryl enzyme. Then you keep adding malonyl enzyme units to build up 2 plus 2 plus 2 plus 2. If you look for it you will find it, but this is a completely random, unselective synthesis. We have run this thing out to where we synthesize fatty acids with 40 carbons, but these fatty acids do not exist in the intact organism; that is all there is to it. The organism synthesizes the specific fatty acids that it needs, and C_{26} is the one that it wants. I think that even though Dr. Lederer is speculating, as far as the *in vivo* synthesis of mycolic acid is concerned, I am forced to admit that I suspect he is right. It seems incredible, but, on the basis of absolutely no *in vivo* information whatsoever, he has proposed a synthesis that I suspect is going to prove absolutely correct.

Finally, Dr. Kallio's work fits in with ours in a way that I believe is quite remarkable. In order to get a condensation of fatty acids, in other words a synthesis of long-chain fatty acids by the condensation of medium-chain fatty acids, it is impossible to devise a mechanism that does not go through omega oxidation of fatty acids. Now this is a concept that up to a couple of years ago was strict heresy in contemporary biochemistry. However, the oxidation of alkanes has been demonstrated, and very recently some highly respectable people have shown that omega oxidation of fatty acids does occur. While there is some controversy as to the mechanism of this oxidation the fact remains that you can oxidize the methyl end. The work from alkanes has come from Dr. Kallio, from Dr. Coon and from Dr. Senez. There is absolutely no question about this. I think what we have shown is that in the myco-

bacteria the significance of oxidation of the methyl end of an alkane is that this is the intimate mechanism of the synthesis of complex fatty acids.

Dr. Long. Thank you for tying that together. I am going to call on one more speaker. Dr. Segal, would you care to comment on some of the questions raised.

Dr. Segal. I brought several slides with me in compliance with the invitation to participate in this conference. But since our discussion time has run out, perhaps I can summarize briefly their relevance to the question Dr. Tepper raised regarding the methodologic validity of lipid analysis of *in vitro* grown pathogenic mycobacteria as representative of the *in vivo* condition. For a number of years now I have been investigating a wide range of differential characteristics, such as biochemical, pathogenic, immunogenic, of *in vitro* grown *M. tuberculosis* strain H37Rv and of the same strain grown *in vivo*, separated mechanically from the lungs of tuberculous mice by a method of differential centrifugation. Furthermore, a comparative study of lipid composition, in collaboration with Father Miller of Regis College in Denver, has revealed significant differences between these two states of growth of the tubercle bacillus.

TABLE 2. *Comparative lipid composition of* in vivo *and* in vitro *grown* M. tuberculosis (*using the methods of column chromatography and thin layer chromatography applied to alcohol-ether extracts*).

Fraction	Percentage	
	In vivo	*In vitro*
Hydrocarbons	14.3	11.4
Triglycerides	25.9	9.3
Free fatty acids	10.0	11.4
Di- and mono-glycerides	19.9	5.6
Polar lipids (phospholipids and lipoproteins)	29.9	62.3

son and associates, several significant differences were found to exist between the *in vivo* and *in vitro* grown organisms. The *in vivo* grown bacilli contain an even greater lipid concentration (close to 50 per cent of dry weight), a result seemingly paradoxic in view of their hydrophilic character in contrast to the characteristic hydrophobic behavior of *in vitro* cultured tubercle bacilli. A statistically significant greater content of ether-alcohol and bound lipid fractions is present in the *in vivo* bacillus, whereas its chloroform soluble fraction was found to be about one-half that of the *in vitro* grown bacillus (Table 1).

TABLE 1. *Comparative lipid composition of* in vivo *and* in vitro *grown* M. tuberculosis (*using the method of solvent fractionation*).

Organism	Dried extract. Per cent of dry weight of bacilli			
	Ether-alcohol	Chloroform	Ether-alcohol + 1% HCl	Total lipid
In vitro	16.2	7.4	15.8	39.4
In vivo	24.5	3.9	20.7	49.1

Whereas tubercle bacilli grown *in vitro* characteristically fix neutral red and are not acetone-fast after Sudan black B staining, the mouse-lung-separated tubercle bacilli do not fix neutral red (although they have twice the virulence of the *in vitro* bacilli) and are acetone-fast after Sudan black B staining. On employing the classic method of solvent extraction of mycobacterial lipids as originally outlined by Anderson and associates, several significant differences were found to exist between the *in vivo* and *in vitro* grown organisms. The *in vivo* grown bacilli contain an even greater lipid concentration (close to 50 per cent of dry weight), a result seemingly paradoxic in view of their hydrophilic character in contrast to the characteristic hydrophobic behavior of *in vitro* cultured tubercle bacilli. A statistically significant greater content of ether-alcohol and bound lipid fractions is present in the *in vivo* bacillus, whereas its chloroform soluble fraction was found to be about one-half that of the *in vitro* grown bacillus (Table 1).

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 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.

These studies of lipid composition have recently been extended by use of the methods of silicic acid-column chromatography and thin layer chromatography. Results are in a preliminary state. One unexpected result is the finding of a two-fold greater percentage of polar lipids for the *in vitro*-grown organism. The hydrophilic character of the *in vivo*-grown bacilli had been assumed to be due to a higher concentration of polar lipids. Since this is not the case, alternative hypotheses will be explored (Table 2).

It is not possible at this point to interpret these differences in lipid composition in specific chemical and biologic terms. However, the finding of a low chloroform-soluble lipid content of *in vivo*-grown tubercle bacilli is consistent with recent immunologic findings indicating that the *in vivo* organism exhibits a lower immunizing capacity against acute tuberculous infection in mice (using a variety of vaccine preparations), as well as a diminished capacity both to induce and to elicit a delayed hypersensitivity response in rabbits and guinea pigs.

These results clearly point up the need for continued studies of *in vivo*-grown *M. tuberculosis*, as well as offer a supportive basis for applying such a methodologic approach to the study of *M. leprae* and *M. lepraemurium*. This is not to overlook the daring efforts at this approach made several years ago by Dr. Hanks and Dr. Gray, but only to urge continuation of their pioneering research along these lines.

Dr. Long. Thank you very much. I think we can close by saying simply that the tubercle bacillus probably does know its own business and what it is trying to do, and I think that holds for *M. leprae* also. As a final remark I might say that I agree thoroughly that we should know something of the composition of mycobacteria as they occur in tissues; it would seem to me that a disease such as lepromatous leprosy in which, as I have been told, one-third of what you see before you is made up of mycobacteria, might furnish an unusual basic material on which chemical analyses could be made of organisms that grew *in vivo*.