STUDIES OF THE UTILIZATION OF C\textsuperscript{14}-LABELED OCTADECENOIC ACIDS BY
LACTOBACILLUS ARABINOSUS\textsuperscript{1}

WILLIAM M. O'LEARY\textsuperscript{2}

Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Illinois

Received for publication September 25, 1958

The ability of long chain unsaturated fatty acids to replace biotin in the nutrition of various
members of the genus Lactobacillus has been well established; however, the exact nature of this
biotin-fatty acid relationship is in dispute, and a number of conflicting explanations have been
suggested for this phenomenon (Nieman, 1954). Williams et al. (1947) were among the first to
suggest that in these bacteria biotin may be involved in some way in the biosynthesis of certain
indispensable fatty acids, and that when such acids are supplied preformed to the bacteria the
biotin requirement is minimized. This concept has come to be widely regarded as the most
plausible of the hypotheses thus far advanced to explain the biotinlike activity of unsaturated fatty
acids, and has been found to be reconcilable with a variety of experimental observations in-
cluding antimetabolite effects and the alterations in bacterial lipide spectra caused by changes
in the fatty acid content of media (Nieman, 1954; Deuel, 1957; Hofmann et al., 1957). Implicit in
and essential to the validity of this hypothesis is the ability of lactobacilli to incorporate into their
cells and to further utilize fatty acids supplied in media in lieu of biotin. Until the present time
this ability has of necessity been tacitly assumed on the basis of inference from indirect evidence.

Although a number of unsaturated fatty acids, of which oleic acid is best known, have been
shown to exhibit biotin-replacing capabilities (Axelrod et al., 1948; Kodicek, 1949; Cheng et al.,
1951; Hofmann and Panos, 1954; O'Leary and Hofmann, 1957), it has been found that cis-
vaccenic (cis-11,12-octadecenoic) acid is the major unsaturated fatty acid component of the
 cellular lipides of lactobacilli (Hofmann, 1953).

This acid has also been shown to mark a bacteriophaga biotin-replacing capability (Cheng et al.,
1951; Hofmann and Panos, 1954). Further studies by Hofmann and his co-workers (1957) led them to
suggest that cis-vaccenic acid may be the precursor of lactobacillic (cis-11,12-methylene-
 octadecanoic) acid, another biotin-replacing compound found in appreciable amounts in lipides of lactobacilli.

The purpose of the investigation reported here was to determine whether or not cis-vaccenic and
oleic acids are actually incorporated into bacterial cells when supplied in media in lieu of biotin, and if so, whether they are retained as such or are converted into other lipoidal ma-

MATERIALS AND METHODS

Bacteria. Lactobacillus arabinosus strain 17-5, ATCC 8014, was employed for all studies of octa-
decenoic acid utilization. Lactobacillus delbrueckii ATCC 9649 was used for the microbiological
assays of both unsaturated acids and saturated C\textsubscript{19} acids. Both organisms were maintained as
stab cultures in medium of the following percentage composition: yeast extract, 1.2; glucose, 1.0;
sodium acetate, 0.8; agar, 2.0; pH, 6.8. Cultures were transferred weekly, incubated 36 hr at 35 C, and stored at refrigerator temperature.

Fatty acids. Oleic-1-C\textsuperscript{14} acid was obtained from Tracerlab, Inc., Waltham, Massachusetts.
Since chromatographic analysis of a sample of this acid revealed no chemical or radiochemical
impurities, it was used without further purification. For use in preparing culture media, suitable
specific activities were obtained by adding unlabeled oleic acid of high purity to the oleic-1-C\textsuperscript{14}
acid.

cis-Vaccenic-1-C\textsuperscript{14} acid was synthesized in this laboratory. 1-Chloro-10-heptadecyne was pre-
pared and purified using the methods of Ahmad et al. (1948), and carbonated by the method
described by Calvin et al. (1949) using BaC\textsubscript{14}O\textsubscript{3}. The 11,12-octadecenoic-1-C\textsuperscript{14} acid so obtained
was purified by successive fractional recrystallizations from petroleum ether (30 to 60 C), from
ethanol, and from acetone. This acetylenic acid
TABLE 1
Composition of the semisynthetic medium used for the cultivation of Lactobacillus arabinosus

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Casein hydrolyzate*</td>
<td>50.0 ml</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>100.0 mg</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>100.0 mg</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>100.0 mg</td>
</tr>
<tr>
<td>Adenine sulfate</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Guanine hydrochloride</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Uracil</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Xanthine</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Thiamin hydrochloride</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Niacin</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>0.05 mg</td>
</tr>
<tr>
<td>K2HPO4</td>
<td>500.0 mg</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>500.0 mg</td>
</tr>
<tr>
<td>MgSO4·7H2O</td>
<td>200.0 mg</td>
</tr>
<tr>
<td>FeSO4·7H2O</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>MnSO4·H2O</td>
<td>6.0 mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Octadecenoic acid†</td>
<td>30.0 mg</td>
</tr>
<tr>
<td>Tween 40</td>
<td>1.0 g</td>
</tr>
<tr>
<td>pH</td>
<td>6.6-6.8</td>
</tr>
</tbody>
</table>

* Casein Hydrolyzate, 'Vitamin Free', Acid Hydrolyzed, Liquid, General Biochemicals, Inc., Chagrin Falls, Ohio.
† The fatty acid was dissolved in the Tween 40 and the resultant solution was then added to the bulk of the medium.

was then partially hydrogenated using palladium-on-charcoal catalyst in ethanol containing 20 per cent pyridine as described by Hofmann and Sax (1953). The resulting cis-vaccenic-1-C14 acid was recovered from the hydrogenation mixture and purified by fractional recrystallization from the three solvents mentioned above followed by a reversed-phase partition chromatography technique described by Aronoff (1956). This acid was prepared with a specific activity low enough (i.e., approximately 94,000 cpm per μmole) to permit its use without dilution with unlabeled acid. Its physical and chemical characteristics as well as those of the intermediates in its synthesis were essentially the same as those reported by Ahmad et al. (1948).

Culture methods. For all cultures of L. arabinosus in which cis-vaccenic-1-C14 or oleic acid was substituted for biotin, a modification of the medium of Wright and Skeggs (1944) was employed (table 1). To prepare an inoculum, a tube containing 15 ml of this medium was inoculated from a stock culture and incubated 24 hr at 35 C. The cells were recovered by centrifugation and washed with five 20-ml portions of 0.85 per cent sodium chloride solution. An aliquot of 0.1 ml of the final suspension was used to inoculate another tube of the same medium. Three successive transfers were made in this manner. Two-mI aliquots of the final saline suspension of the third culture were used to inoculate each of three Erlenmeyer flasks each containing 500 ml of medium. After incubation for 36 hr at 35 C, the contents of each flask were added aseptically to one of three culture bottles each containing 4 L of similar medium. These bottles were also incubated 36 hr at 35 C. In preparing media for the flasks and bottles, acids of the following specific activities were used: cis-vaccenic-1-C14, 93,700 cpm per μmole; oleic-1-C14, 50,150 cpm per μmole.

After incubation, the cells in the bottle cultures were collected by centrifugation and washed by repeated suspension and centrifugation using a total volume of 5 L of distilled water. The collected cells were then lyophilized and stored in vacuo over phosphorus pentoxide.

Extraction and analysis of cellular fatty acids. After weighing, the dried cells were hydrolyzed by autoclaving with 100 ml of 2 N sulfuric acid for 1½ hr at 121 C. The cellular fatty acids were extracted from the hydrolyzate, subjected to a hydroxylation procedure that converted the unsaturated fatty acids present to their chromatographically separable dihydroxy derivatives, and analyzed by rubber column chromatography using the techniques of Hofmann et al. (1955). The acetone-water chromatographic eluate fractions were monitored by titration with 0.01 N NaOH.

The individual fatty acid present in each chromatographic peak was recovered by acidification and ether extraction of the eluate after removal of the acetone under reduced pressure. After washing, each ether extract was dried and the ether was removed under reduced pressure.

For the determination of specific activities, weighed aliquots of each fatty acid residue were dissolved in 10.0 ml of ethanol. Aliquots of 100 μL of each ethanolic solution were mixed with 10.0 ml of scintillation fluid consisting of 0.4 per cent 2,5-diphenyl-oxazole dissolved in a mixture...
of 70 per cent toluene and 30 per cent ethanol. The radioactivies of these mixtures were then measured using a Packard "Tri-Carb" automatic scintillation counter.

Microbiological assays. The assay techniques employing L. delbrueckii described by Hofmann et al. (1955) were used to evaluate the C19 chromatographic fractions and to determine the amounts of octadecenoic acid remaining in media after removal of bacterial cells.

RESULTS

Utilization of cis-vaccenic-1-C14 acid. From the culture in which cis-vaccenic-1-C14 acid was supplied in lieu of biotin, a total of 7.0 g of dried bacterial cells were obtained from which 165.9 mg of fatty acids were extracted.

Microbiological assay of the medium after growth showed that of the 405 mg of cis-vaccenic acid originally present, only 300 mg remained, indicating that 105 mg or 0.374 mmole had been utilized during growth of the bacteria.

The various fatty acids present in the bacteria were determined chromatographically. A typical chromatographic pattern is shown in figure 1, and table 2 presents the results of these analyses calculated on the basis of unhydroxylated cellular fatty acids.

![Figure 1](http://jb.asm.org/)

**Figure 1.** Typical chromatographic pattern of the fatty acids of Lactobacillus arabinosus grown on cis-vaccenic-1-C14 acid (48-mg sample). Elution solvents (A35, etc.) were composed of acetone-water mixtures containing the volume per cent acetone indicated by the subscript number.

**TABLE 2**

Analysis in duplicate of fatty acids from Lactobacillus arabinosus grown on cis-vaccenic-1-C14 acid (83,700 cpm per mmole). Values are calculated on the basis of unhydroxylated cellular fatty acids

<table>
<thead>
<tr>
<th>Acid</th>
<th>Cellular Acids</th>
<th>Specific Activity*</th>
<th>Total Cellular Content</th>
<th>Biotin Activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>cpm/µmole</td>
<td>mmole</td>
<td></td>
</tr>
<tr>
<td>Octadecenoic</td>
<td>22.4</td>
<td>22.6</td>
<td>89,100</td>
<td>0.132</td>
</tr>
<tr>
<td>Capric</td>
<td>1.9</td>
<td>2.0</td>
<td>—</td>
<td>0.133</td>
</tr>
<tr>
<td>Lauric</td>
<td>3.0</td>
<td>3.0</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Myristic</td>
<td>2.6</td>
<td>2.4</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Palmitic</td>
<td>29.8</td>
<td>30.1</td>
<td>890</td>
<td>0.222</td>
</tr>
<tr>
<td>Lactobacillie</td>
<td>39.6</td>
<td>39.4</td>
<td>88900</td>
<td>0.221</td>
</tr>
<tr>
<td>Totals</td>
<td>99.3</td>
<td>99.5</td>
<td>3.54</td>
<td>15.2</td>
</tr>
</tbody>
</table>

* = Not significantly above background.
† Expressed as µg of biotin per mg of fatty acid. This was found to be 15.2 for an authentic sample of lactobacillie acid.
Microbiological assay of the material recovered from the C_{19} chromatographic peak indicated that it was composed entirely of lactobacillic acid.

It has been reported that this technique may not detect small amounts of stearic acid which, if present, would also appear in this peak (Hofmann et al., 1955); however, the neutral equivalent (294) and the melting range (27.7 to 29.0°C) of this material were also found to agree with published values for lactobacillic acid (Hofmann and Lucas, 1950). Consequently, for purposes of calculations the material in the C_{19} fraction was treated as consisting solely of lactobacillic acid.

The necessity of converting the cellular octadecenoic acids to their biologically inactive dihydroxy derivatives in order that they might be chromatographically resolved from analogous saturated fatty acids precluded microbiological assay of this fraction (C_{19} Di-OH). However, mixed melting point determinations with authentic samples of 9,10-dihydroxy-octadecanoic acid and 11,12-dihydroxy-octadecanoic acid indicated that the hydroxylated cellular acid was the 11,12 isomer, i.e., the derivative that would be obtained from cis-vaccenic acid.

These analyses showed that the lipides of bacteria grown on cis-vaccenic-1-C\textsuperscript{14} acid in lieu of biotin contained a combined total of 0.354 mmole of octadecenoic and lactobacillic acids, or approximately 95 per cent of the amount of acid apparently removed from the medium by the bacteria.

Measurements of the specific activities of the various fatty acid fractions from the cells of *L. arabinosus* grown on cis-vaccenic-1-C\textsuperscript{14} acid showed that most of the radioactivity was located in the octadecenoic and lactobacillic acids. The activities of both of these acids were approximately 95 per cent of that of the cis-vaccenic-1-C\textsuperscript{14} acid supplied in the medium. Capric, lauric, and myristic acids and the nonsoapifiable matter obtained in the process of extracting the cellular fatty acids were not found to contain any significant amount of radioactivity, and palmitic acid contained only a small amount.

**Utilization of oleic-1-C\textsuperscript{14} acid.** Dried cells (5.8 g) were obtained from the culture containing this acid in lieu of biotin, and from these cells, 132.1 mg of fatty acids were extracted.

Microbiological assay of the medium after growth showed that of the 405 mg of oleic acid originally present, only 317 mg remained, indicating that 88 mg or 0.312 mmole had been utilized during growth of the bacteria.

Results of the analyses of the fatty acids from these bacteria calculated on the basis of unhydroxylated cellular acids appear in table 3. The findings are generally similar to those in the cis-vaccenic acid study but with quantitative differences. The lipides of bacteria grown on oleic acid contained a combined total of 0.272 (0.271) mmole of octadecenoic and lactobacillic acids or approximately 87 per cent of the amount of acid apparently removed from the medium by the bacteria.

Mixed melting point determinations as mentioned above using the dihydroxy-octadecanoic acid from these oleic acid grown cells indicated that these bacteria also contain the 11,12 isomer of octadecenoic acid. Microbiological assay

<p>| TABLE 3 |
| Analysis in duplicate of fatty acids from Lactobacillus arabinosus grown on oleic-1-C\textsuperscript{14} acid (50,150 cpm per \mu mole). Values are calculated on the basis of unhydroxylated cellular fatty acids |</p>
<table>
<thead>
<tr>
<th>Acid</th>
<th>Cellular Acids</th>
<th>Specific Activity* ( \text{cpm/\mu mole} )</th>
<th>Total Cellular Content ( \text{mmole} )</th>
<th>Biotin Activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octadecenoic</td>
<td>26.6</td>
<td>27.1</td>
<td>42,630</td>
<td>42,400</td>
</tr>
<tr>
<td>Capric</td>
<td>1.5</td>
<td>2.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lauric</td>
<td>2.8</td>
<td>2.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Myristic</td>
<td>2.9</td>
<td>3.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Palmitic</td>
<td>32.2</td>
<td>33.1</td>
<td>990</td>
<td>1,010</td>
</tr>
<tr>
<td>Lactobacillic</td>
<td>33.2</td>
<td>32.1</td>
<td>41,820</td>
<td>41,570</td>
</tr>
<tr>
<td>Totals</td>
<td>99.2</td>
<td>99.7</td>
<td>48,110</td>
<td>48,030</td>
</tr>
</tbody>
</table>

* — = Not significantly above background.

† Expressed as \( \mu \)g of biotin per mg of fatty acid. This was found to be 15.2 for an authentic sample of lactobacillic acid.
indicated that the C₁₉ material recovered from these bacteria is lactobacillic acid.

Measurements of the specific activities of the fatty acid fractions from cells grown on oleic-1-C¹⁴ cells showed that, as was found in the cis-vaccenic-1-C¹⁴ acid grown cells, most of the radioactivity was located in the octadecenoic and lactobacillic acids with little or none appearing in other lipid fractions. However, the activities of the octadecenoic and lactobacillic acids from oleic grown cells were approximately 85 per cent of that of the oleic-1-C¹⁴ acid supplied in the medium, which is somewhat lower than the value found in the cis-vaccenic-1-C¹⁴ acid studies.

**DISCUSSION**

The results of the studies with both cis-vaccenic-1-C¹⁴ acid and oleic-1-C¹⁴ acid provide strong evidence for the concept that octadecenoic acids capable of replacing biotin in bacterial nutrition are actually incorporated into the bacterial cell and constitute the major cellular content of such acids.

These observations support the suggestions of Williams et al. (1947), Potter and Elvehjem (1948), Rubin and Scheiner (1949), and others that biotin is involved in the synthesis of unsaturated fatty acids essential to the cell, and that supplying such acids to the cell will consequently decrease the biotin requirement. This possible function of biotin is especially intriguing, for, although considerable information is available regarding the biosynthesis of straight chain saturated fatty acids, little is known about the formation in bacteria of long chain unsaturated acids such as cis-vaccenic acid, or of cyclopropane ring-containing acids such as lactobacillic acid. Of interest in this respect is the report of the author (O'Leary and Hofmann, 1957) showing that a number of shorter chain unsaturated fatty acids (C₁₆, C₁₇, and C₁₈) are also capable of replacing biotin to varying degrees in the nutrition of lactobacilli. It was suggested that such acids may be intermediates in the bacterial biosynthesis of cis-vaccenic acid by a pathway in which some early steps may be biotin-dependent. Experiments employing isotopic methods similar to those used in the present investigation may be of value in further investigations of this postulated pathway. Wakil et al. (1958) have recently reported on studies with C⁴¹-labeled acetate which produced evidence for the participation of biotin in the synthesis of fatty acids by enzyme preparations from avian liver.

In both cis-vaccenic and oleic acid studies it was noted that the lactobacillic acid had a specific activity similar to that of the octadecenoic acid found in the same cells. This may be interpreted as evidence that lactobacillic acid is formed primarily, if not entirely, from cellular octadecenoic acid. This conversion was originally suggested by Hofmann et al. (1957) on the basis of alterations caused in bacterial lipide composition by changes in the fatty acid content of media. It is still not known, however, what reactions are involved in the apparent addition of a methylene group across the double bond of cis-vaccenic acid to form the cyclopropane ring characteristic of lactobacillic acid.

In the cellular lipide fractions from cells grown on either octadecenoic acid it was observed that radioactivity was largely limited to octadecenoic and lactobacillic acids. This indicates that there was little degradation and redistribution of the carboxyl end of the acid supplied in the medium among other lipide components of the cell.

The results of the cis-vaccenic acid study showed that approximately 95 per cent of the acid missing from the medium after growth could be accounted for by the combined amounts of cellular octadecenoic and lactobacillic acids. If the remaining 5 per cent is not merely ascribable to limitations of the techniques employed, it may represent octadecenoic or lactobacillic acids degraded or converted into nonlipide material. It might be mentioned here that the metabolic fate of lactobacillic acid is as yet quite unknown. It was also found that the cellular octadecenoic and lactobacillic acids exhibited specific activities that were approximately 95 per cent of that of the cis-vaccenic-1-C¹⁴ acid supplied in the medium. This small decrease in specific activity may be due to a low level of de novo synthesis or to some type of exchange reaction.

In the oleic acid study, approximately 87 per cent of the acid missing from the medium could be accounted for by the combined amounts of cellular octadecenoic and lactobacillic acids. The specific activities of the cellular acids were approximately 85 per cent of that of the oleic-1-C¹⁴ acid supplied in the medium. The quantitative differences between the results of the cis-vaccenic acid and oleic acid studies presumably reflect some difference in the bacterial utilization of
these two acids. This may be associated with the metabolic mechanisms involved in dealing with these positional isomers.

Microbiological assay results and physical data indicated that the C₉₉ fractions in both studies consisted of only lactobacillic acid. As was pointed out, if any stearic acid were present it would also be in this chromatographic fraction. The fact that stearic acid was not detected in these cells is in accord with the reports of other workers. Using fractional distillation techniques, Hofmann et al. (1952) found that stearic acid accounted for not more than 2 per cent of the fatty acids isolated from L. arabinosus grown on biotin. In a later study using techniques similar to those employed here, no stearic acid was detected in lipides from the same organism again grown on biotin (Hofmann et al., 1955). Further, it has been shown that growing lactobacillic on unsaturated fatty acids in lieu of biotin tends to markedly decrease cellular stearic acid content and increase lactobacillic acid content (Hofmann et al., 1957).

The results of mixed melting point determinations involving the dihydroxy derivatives of cellular octadecenoic acids, while by no means conclusive, do suggest an interesting field for further investigation. The indication that supplying the 11,12 isomer of octadecenoic acid in the medium in lieu of biotin results in cells containing 11,12 octadecenoic and 11,12-methylene-octadecanoic acids was not unexpected since it has been shown that "normal" cells of L. arabinosus (i.e., grown on biotin) contain these 11,12 isomers (Hofmann et al., 1952). The preliminary indication that cells grown on oleic (cis-9,10-octadecenoic) acid also contain the 11,12 isomers was more interesting. This would suggest that the cells may be capable of isomerizing the 9,10 acid to the 11,12 configuration. More detailed chemical studies on larger samples of acids from bacteria grown on unsaturated fatty acids are, however, required on this point.

While the chromatographic technique used in this investigation for the analysis of cellular fatty acids is the best available up to the present time for this type of work, it necessitates the hydroxylation of unsaturated fatty acids before they can be clearly separated from saturated acids of similar chain lengths. This not only requires considerable time for the necessary manipulations, but, more important, alters the chemical nature of the unsaturated acids resulting in biologically inactive compounds. It is hoped that in future studies it will be possible to adapt gas-liquid chromatography for such work. If suitable separation can be achieved, such a technique should afford rapid analysis of small samples while permitting the recovery of components with unaltered biological activity.

ACKNOWLEDGMENT

The author is indebted to Dr. John H. Pomeroy of this laboratory for his helpful advice regarding the synthesis of cis-vaccenic-1-C¹⁴ acid.

SUMMARY

Lactobacillus arabinosus has been grown in media in which cis-vaccenic-1-C¹⁴ or oleic-1-C¹⁴ acid was supplied in lieu of biotin. The constituent fatty acids of cells so grown were extracted, separated chromatographically, and examined for radioactivity. It was found that most of the fatty acid removed from a medium could be accounted for by the combined amounts of octadecenoic and lactobacillic acids present in cells grown in that medium. Radioactivity in cellular lipide fractions was largely limited to these same two acids. The significance of these observations to biotin-fatty acid interrelationships and mechanisms of fatty acid biosynthesis are discussed.

REFERENCES


