Membrane Fatty Acid Composition As a Function of Growth Temperature

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# Introduction

Here’s one for you: poikilothermic (Greek: "poikilos" *ποικίλος* = "varied," "thermia" *θερμία* = "heat"). A poikilothermic organism is one whose temperature tends to match the temperature of its surroundings. Examples are bacteria, fungi, algae, and plants. The composition of the cell membrane of these organisms varies, depending on the temperature at which they grow. The change in composition with temperature has the effect of keeping the membrane’s viscosity constant. This is called (drum roll) homeoviscous adaptation (homeo- is a combining form meaning “like”, or “similar”). Homeoviscous adaptation presumably allows the proteins in the cell membrane to function over a range of temperatures.

Typically, as temperature increases, the fraction of long-chain and saturated fatty acids increases, as the fraction of short-chain and unsaturated, lower melting point fatty acids decreases. Also, at higher temperatures cyclopropane rings form, presumably by addition of methylene to the double bonds of unsaturated fatty acids. This was first observed in *E. coli*, which is the organism that will be examined in this lab.

The source of the fatty acids is phosphoglycerides, sphingolipids and glycolipids in the cell membrane. Hydrolysis of these biomolecules breaks them into their component parts. Sphingolipids and glycolipids produce one fatty acid each; phosphoglycerides produce two.

# [Above can be CC’ed]

# Determining the Fatty Acids Present in the Cell Membrane

The fatty acids will be separated, identified, and the amounts present determined with a gas chromatography-mass spectrometry, GC-MS. The sample size required by the GC-MS is very tiny, so small amounts of biological material will generate enough material for analysis. GC-MS requires that the species to be analyzed be volatile, because the GC requires that they be in the gas phase. However, fatty acids are not very volatile, because they are polar and can hydrogen bond. Also, the polar hydrogen bonding of the carboxyl group causes the peaks to get broader. These problems are overcome by converting the fatty acids to methyl esters, which are more volatile than the original acids; these FAMEs (fatty acid methyl esters) are then injected into the GC-MS for analysis.

# Procedure

## Preparation of the FAMEs

Figure 1 gives a summary of the procedure.

Centrifuge cell culture & pour off supernatant

Resuspend in 2.5 mL

methanol:toluene (4:1)

Add small stirring bar

& 200 µL of acetyl

chloride; transfer to a glass screw cap vial; cap tightly and heat in a boiling water bath for 60 minutes.

Neutralize with Na2CO3. Transfer to a centrifuge tube, add 1.0 mL toluene, and mix

Centrifuge; transfer top

layer into GC-MS sample vial; 1 L will be inject in the instrument

Figure 1. Protocol for preparation of fatty acid methyl esters

NOTE: Each group will have one of two samples of bacteria grown in Luria-Bertani (LB) broth. One sample will be a culture of bacteria grown between 20 and 25oC. The second sample will be a culture of the same bacteria grown at 40oC. The following procedure is used with both samples.

Take 10 mL of a solution of suspended cells and centrifuge in a 15 mL polypropylene centrifuge tube at 4,000 rpm for 15 minutes; discard the supernatant (the more that is removed, the better the yield of FAMEs will be).

Re-suspend the pellet in methanol-toluene (4:1 v/v) (~2.5 mL total, 2 “squirts” with a Pasteur pipette). Using an autopipet, add 200 L of acetyl chloride a drop at a time, swirling between additions.

When acetyl chloride reacts with alcohol or water, it produces so much heat that it sizzles when it hits the surface of the liquid. Therefore, add this cautiously a drop at a time.

The acetyl chloride is a catalyst for the “transesterification” reaction that will produce fatty acids directly from the membrane lipids. The methanol provides the methyl group for the formation of the methyl esters.

With a plastic disposable pipet transfer all of the solution to a screw-cap glass sample vial. (The plastic centrifuge tubes do not seal tightly enough to hold the volatile methanol-toluene mixture at 100°C.)

 Add a small magnetic stirring bar. Tightly close the sample vial. Place the vial in a boiling water bath on a magnetic stirrer-heater and let it react for 60 minutes. (The magnet will stir when the tube is suspended in the water bath.)  **Do Not** put the tube directly on the bottom of the beaker containing your water bath - the temperature in contact with the bottom of the beaker is higher than 100oC and the solvent will vaporize.

Cool the tube in water, then transfer the solution back into the centrifuge tube. Add 5 mL of 6% Na2CO3 solution slowly to the contents of the tube. Swirl to mix; some CO2 is expected to be released. Then put the lid on and shake to thoroughly mix the contents.

Next, extract the fatty acids from the aqueous solution into the toluene. To do this, add 1 mL of toluene, cap the tube tightly, and shake the tube vigorously. Balance against another tube and centrifuge for 5 min at 4000 rpm in the large centrifuge.

The upper toluene layer will contain the derivatized fatty acids from the membrane. Using a Pasteur pipette, transfer most of this layer to one of the small sample vials for the GC-MS autosampler. Label the vial with the initials of those in your group, and the temperature at which the *E. coli* was grown, either RT or 40°C.

## GC-MS Report (Provided next lab period)

The toluene layer will be analyzed by GC-MS, and a report will be printed out. The report starts with the GC chromatogram for the sample, Figure 2. Following this (not shown) is a list of each peak’s, retention time and area. The list is limited to no more than the 20 largest peaks. Notice the excellent resolution that is obtained - even peaks with only slight variations in chemical structure are well resolved. Some of the peaks correspond to specific fatty acid methyl esters; others are other materials that were in the sample, which can probably be ignored.



Figure 2. GC chromatogram of *E. coli* membrane FAMEs

The report also includes the mass spectrum for each peak, and the mass spectrum from the computer’s library that best matches that peak. The upper trace will be the experimental mass spec and the lower trace will be the library’s best match, including a picture of the structure. Figures 3A and 3B show you the mass spectra of two slightly different methyl esters that are found in the membranes of *E.coli*.



### Figure 3A



### Figure 3B

The “Best match” entry includes a “similarity index”, such as 97/100, which is a measure of how good the fit is. Any fit below 85% is likely an incorrect match.

Area % designates the percentage of this individual substance as a part of all the components that were separated by the GC. Use this information for tabulating the saturated and unsaturated FAMEs.

# Analysis

In the peak list in the GC-MS report, identify the methyl esters. Working with a group that analyzed the *E. coli* grown at the other temperature, prepare a table like so:

|  |  |  |
| --- | --- | --- |
|  | **RT** | **40°C** |
|  | Similarity Index | Area % | Similarity Index | Area % |
| **Saturated FAMEs** |  |  |  |  |
| Name of one |  |  |  |  |
| Name of another |  |  |  |  |
| **Unsaturated FAMEs** |  |  |  |  |
| Name of one |  |  |  |  |
| Name of another |  |  |  |  |
| (list all that are present) |  |  |  |  |

Write a paragraph discussing how the results are consistent (or not) with the expected results. Note: at higher temperatures, some cyclopropane rings may be present; comment on that, if observed. Are there different fatty acids present in the two samples? Do the amounts (Area%) of those fatty acids common to both samples change with temperature? Is there an increase in the relative abundance of saturated (or unsaturated) fatty acids with temperature? How about a change in the abundance of longer chained fatty acids? Does the saturated to unsaturated ratio change for a given chain length? Do your results support the general notion that organisms adjust their membrane composition to maintain a constant “fluidity”?

# References

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*LB Broth Recipe*
*(Luria-Bertani)*

**Tryptone** is a peptide formed by the digestion of casein (commonly found in milk) by the protease trypsin. Tryptone is commonly used in microbiology to produce Lysogeny broth for the growth of *E. coli*. It provides a source of protein for the growing bacteria.

# Materials

Used to prepare bacteria:

bacteria: *E. Coli* strain

water bath at room temperature (22 to 25oC)

water bath at 40oC

LB Broth, 200 mL for 20 students, so about 500 mL for two sections. To prepare, place 12.5 g of LB Broth powder in a 1 L Erlenmeyer flask. Add 500 mL deionized water. Place an upside-down beaker over the top of the flask to keep out bacteria. Heat to boiling. Pour half of the solution into a 500 mL Erlenmeyer flask, then cover that flask with an upside-down beaker, also. So as not to kill the *E. coli*, cool the contents of the two flasks (running water, or ice work well). The 1 L Erlenmeyer should be cooled to room temperature; the 500 mL flask will be maintained at 40°C in a water bath. Put about equal amounts of the *E. coli* solutions into the two flasks.

Used for the lab:

Beckman centrifuge (the large centrifuge in the organic lab)

15 mL polypropylene centrifuge tube, 2 per student

methanol:toluene (4+1 v/v), 50 mL per 20 students, so need 100 mL

toluene, about 20 mL for 20 students, so a 100 mL bottle would do

culture tube with Teflon lined cap (also called a sample vial) Need the large ones with black lids, because they need to hold about 9 mL of solution.

small magnetic stir bar to go in the culture tube

stirrer/hot plate

acetyl chloride; 4 mL for 20 students, so need about 5 mL (a large bottle will do)

100 L glass syringes

400 mL beaker

small glass sample vial for placing the sample in the GC/MS autosampler

gas chromatograph/ mass spectrometer

6% Na2CO3(aq), 5 mL per student, so 100 mL per section, so about 250 mL.

Also need: 2 5 mL autopipettes for dispensing *E. coli*.