A laboratory experiment demonstrating the dynamic character of membranes

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Abstract

An experiment demonstrating changes in the fatty acid composition of bacterial membranes from bacteria grown at 22 and 37 °C is described. The changes in composition confirm expected generalizations. The isolation method is simple and provides samples of fatty acid methyl esters ready for gas chromatography-mass spectroscopy (GC-MS) analysis within about 1.5 h. Analysis by GC-MS involves the use of a computer library for fatty acid methyl ester identification and takes an additional hour. The experiment can easily be adapted to study other factors that influence membrane composition. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

Modern biochemistry laboratory coursework has evolved to focus on the characteristics of proteins and nucleic acids. Given time constraints and the necessity of giving enhanced coverage to areas such as biotechnology, some traditional areas of coverage, for instance, biological lipids have been given less emphasis. When included, lipid experiments often have a natural products/organic chemistry flavor focusing on isolation, characterization and purification. Examples include the isolation of a lipid from nutmeg and the fatty acid content of natural oils described in [1], a widely used experimental biochemistry text. In contrast, the primary content area, by far, in terms of coverage of lipids in standard biochemistry texts, is their dominant presence in membranes. The dynamic nature of membranes is often emphasized. Statements such as the following are made: “bacteria and cold-blooded animals such as fish modify … the fatty acid compositions of their membranes with ambient temperature so as to maintain a constant level of fluidity” [2]. The nature of the changes generally stated in texts involve varying the length and the extent of unsaturation of the fatty acyl chains [2-4]. In fact, bacterial membrane composition is dependent not only on growth temperature, but also growth stage and growth media composition and is referred to as homeoviscous adaptation [5].

One of the foci of our biochemistry course is the importance of weak interactions in explaining biological phenomenon. The character of membranes is a clear demonstration of the importance of van der Waals attractions (London forces) in maintaining the integrity of a biological structure. We have, therefore, incorporated a simple, highly adaptable experiment that supports this focus and, in addition, exposes the student to modern instrumental analysis and use of computer libraries in structure determination. This experiment can be readily accomplished during a 3–4 h laboratory period. In fact, with a little pre-lab work by the instructor, the preparation of samples for analysis can be completed while another experiment, (in our case the isolation of DNA from the same biological source) is started.

2. Materials and methods

When starting from a bacterial source (the most common case), the experiment can be divided into three phases:

- Phase 1 — growth and harvesting of cells.
- Phase 2 — formation of fatty acid methyl esters (FAMEs).
- Phase 3 — GC-MS of FAMEs.

The overall experiment is summarized in Fig. 1 and details for each phase are given below.
2.1. Phase 1

The instructor prepares the cells that will be the source of the FAMES. Any common bacterial culture can be used. We have used the *E. coli* strain JM 109 primarily because we also use this strain for doing transformations. For comparing temperature differences, a 5.0ml starter culture grown at 37°C is used to inoculate 100 ml LB broth cultures at two different temperatures (in the experiment described here, 22 and 37°C). The two cultures are harvested when the absorbance reading at 600nm in a Spectronic Instruments Genesys 2 (or 5) spectrophotometer is between 0.6 and 0.8 corresponding to a cell density of $10^8$ cells/ml. Cell suspensions are divided into 10ml aliquots and centrifuged in 16 ml polypropylene centrifuge tubes at 15,300 g (10,000 rpm with a Beckman JA-14 rotor) for 20 min. After discarding the supernatant, the cell pellet can be used directly or frozen until needed. We have found that by growing, harvesting and then freezing cells, we have much better control over experimental parameters than if we have students to do this. In addition, a significant amount of class time is saved by having the cells ready for formation of FAMES. Instead, we demonstrate with an unharvested culture how pelleted cells are obtained during the course of the lab period. In a typical experiment, the lower temperature cultures provide multiple student samples for FAME analysis. Higher temperature cultures provide for each student (or student group) 10 ml of culture for FAME analysis with the remaining 90 mL being used for isolation of DNA in a parallel experiment.

2.2. Phase 2

A slightly modified procedure for the formation of membrane FAMES as described by Lepage and Roy is used [6]. Thawed cells are resuspended by successive washes of 2.5 ml (total) methanol : toluene (4:1 v/v) with a Pasteur pipette. The resuspended cells are quantitatively transferred to a 15 ml Teflon-lined screw cap culture tube, a 8 mm x 1.5mm magnetic stirring bar is added and the mixture is stirred for an additional minute. The instructor initiates the acid-catalyzed transesterification reaction by slowly adding, with continued stirring, 200 μl of acetyl chloride. The tightly closed culture tubes are placed in a boiling water bath on a magnetic stirrer-heater and allowed to react with moderate stirring for 1 h. During this 1 h reaction time, other experiments, such as a DNA isolation experiment, is started. Students occasionally monitor the reaction to ensure that the solution is not evaporating and replenish the volume with methanol/toluene as needed.

After an hour, the tubes are cooled to room temperature and 5 ml of 6% Na$_2$CO$_3$ is slowly added to neutralize the reaction mixture. An additional 1 ml of toluene is added to the neutralized solution, the mixture is vortex mixed and the contents are transferred to a 15 ml Corex centrifuge tube. The solution is then centrifuged at 1350 x g (3000 rpm with a Beckman JA-14 rotor) for 20 min. A portion of the upper toluene layer is removed and placed in a small vial and stored at 20°C until needed.
2.3. Phase 3

GC-MS analysis of the FAME derivatives can be done in several different ways and is highly dependent on availability of the GC-MS. During the lab period in which this experiment is done, groups of 4–5 students are taken to the GC-MS (there are several “breaks” in the routine that allow this). They are instructed as to the theory and practical aspects of how the instrument works, how to inject the sample, how to use the software, and how to process their results. This takes about 15 min per group and an injection of a student sample is done with commentary interwoven with the display of real-time results. Since students do all their work in our laboratory in teams of three students, one member of the team can do the injection while other team members are involved in other tasks such as DNA isolation and analysis. In our case, we have the instrument all day and the 24 students from 12 teams in two full sections can be done in a long day.

The specifics for the GC-MS setup and run are as follows: Students inject 1 μl of each of their two samples into a Hewlett-Packard 5890 Series II Plus gas chromatograph fitted with a 5972 Series mass selective detector. Instrument operation, data acquisition and analysis is under the control of the instrument’s Chemstation G1701BA Version B.01.00 software. A Supelco MDN-5 bonded poly (5% diphenyl/95% dimethylsiloxane), 30 m × 0.25 mm × 0.25 μm film thickness GC capillary column is used. The injector and detector temperatures are 280°C. The initial temperature of the column is 150°C; after 5 min the temperature is ramped up at 5°C per minute to a final temperature of 225°C at the end of the run (20 min). Helium is used as the carrier gas and the MS operating parameters are: electron multiplier between 1850 and 2000 V, transfer line 280°C, source and analyzer 280°C, optics tuned at m/z 502, electron impact energy = 70 eV. The mass spectral data that are acquired by the instrument are compared to the instrument’s nbs75k MS library. This MS library containing mass spectra data for 75,000 compounds can be obtained from the National Bureau of Standards and is part of the instrument package.

In this experiment we are more interested in the relative changes in the fatty acid composition of the membrane than the characterization of these fatty acids. We, therefore, have the software set up to print out the GC spectrum of each sample followed by a list of the names of the three best computer nbs75k MS library matches for the substance at each retention time. In addition to the mass spectrum identity of the peaks, the relative abundance for each peak is determined as the “identification quality” of the computer match. For data analysis, students are told to ignore peaks with a relative abundance of less than 1% or an identification quality of less than 90%.

3. Results and discussion

Overall, our experimental protocol provides a simple one-pot method. The use of the Lapage and Roy method for direct transesterification of the membrane lipids circumvents lengthy and cumbersome solvent extraction, purification, hydrolysis and derivatization steps. Their rapid and simple method yields high recoveries ( > 98%) of C14–C18 FAMEs [6]. The only difference between the formation of FAMEs in this experiment and theirs is the substitution of methanol: toluene (4:1 v/v) for their methanol: benzene (4:1 v/v) solution. The substitution of toluene for benzene should not affect the chemistry of the transesterification reaction or the extraction of the FAMES, but more importantly, our substitution lessens the concerns for the safety of the students handling these chemicals. As the toluene extraction leads to high recoveries of the FAMES, we are able to bypass the use of the more traditional chloroform/methanol extraction method and avoid the problems of chloroform exposure and waste disposal. The acid-catalyzed transesterification reaction used here is also different than the more common alkaline methanolysis method for the formation of FAMES. The acetyl chloride generates the dry HCl catalyst by a twofold reaction with acetyl chloride with any water present in the methanol and with the methanol itself [7].

Fig. 2 shows an actual result of two chromatograms obtained by a team of students. Students can easily see the qualitative differences between the “FAME profiles” of E. coli grown at the two different temperatures. When comparing the 37°C chromatogram with the 22°C chromatogram, the most dramatic observations are the appearance of new peaks at 11.3 and 15.3 min and the relative abundance changes for the peaks at 12.9, 13.4, and 16.8 min.

The computer’s internal MS library makes identification of peaks easy. Saturated FAMES present no problems. In fact, the computer usually gives multiple mass spectra library matches with 99% confidence (there are multiple mass spectra for common FAMES in the MS library). Unsaturated fatty acids present a slight problem in that different possible positions for unsaturation are given as possibilities for the peak. Although methods exist that can be employed to determine the double-bond position [8], the intent of this experiment is only in monitoring the relative abundance of saturated and unsaturated membrane fatty acids.

In terms of quantification, the area percent for each peak as a portion of the entire spectrum is given as a part of the data output. Since the solvent peak is zeroed out and if all the other peaks are identified as FAMES (which is usually the case), the relative contribution of each fatty acid in the membrane at the given temperature is obtained directly. Boyer [9] points out that different FAMES have different response correction factors for...
a thermal conductivity detector (ranging from 0.908 to 1.172 for common FAMEs). Since our goal is more semi-quantitative and our emphasis is on the change in the magnitude of weak interactions with a change in temperature, we typically do not make these corrections. In some cases when peaks with low confidence values and minor peaks have to be eliminated, relative percentages can be recalculated on the basis of the remaining peaks.

Results obtained by the experiment described here confirm two of the generalizations about the changes in FAME composition with temperature that have been reported in the literature [5,10,11]. The most fundamental generalizations are: (1) there is a decrease in the level of unsaturation with increasing temperature; (2) there is the appearance of odd-chain fatty acids with increasing temperature. Casual inspection of Fig. 3 immediately highlights each of these generalizations. The generalization that there is an increase in chain length with temperature is not apparent from the data. In fact the weighted average saturated chain length for both samples is identical (15.6).

A common method for comparison is to report the unsaturated-to-saturated ratio calculated as the total percentage of 16:1 plus 18:1 FAMEs divided by the total percentage of 14:0 plus 16:0 FAMEs [10]. Table 1 uses this method to compare the results in this experiment with those reported by Marr and Ingraham [5] with E. Coli strain LM30 cells grown in glucose minimal medium at slightly different temperatures.

Although the numbers do not correlate exactly, the trend is the same, that is, as the temperature increases, the ratio of unsaturated-to-saturated FAMEs decreases. Differences in the ratios can be attributed to differences in the growth stage, the growth medium composition [5] and bacterial species or strain [10].

Odd-chain fatty acids are identified by the MS spectra library as the methyl esters of pentadecanoic and heptadecanoic acids. It is possible that these peaks could be the methyl esters of the cyclopropane fatty acids methyl-enetetradecanoic acid and methylenehexadecanoic acid. The latter have been detected in the stationary growth phase of E. coli [5] and Pseudomonas fluorescens [11] and at elevated temperatures for both species of bacteria [11]. A closer analysis of our mass spectra data for the assigned C15 and C17 FAMEs appears to be warranted.
4. Conclusions

This experiment clearly yields results that support the notion that bacteria do change the fatty acid composition of their cell membranes to accommodate the changes in their environment. The experiment can be easily modified from semester to semester to compare other growth temperatures or to investigate other parameters such as age and growth conditions that also affect membrane composition. In addition, the experiment can be used to compare the unique FAME “profiles” of different bacteria that are used for taxonomic purposes. Although uses for GC-MS are becoming more common in an organic setting, GC-MS experiments are still fairly uncommon in mainstream biochemistry courses. Given the emerging significance of this technique in biochemical applications such as protein and nucleic acid sequencing, introduction of this technique in the biochemical context appears to be fully warranted. The additional aspect of this experiment that reflects another trend in science is the application of computing power to simplify and solve problems. The knowledge, use and application of various databases are skills that are now essential for a well-trained biochemist.

The experiment is easily accomplished within a lab period and in fact can be intercalated with other experiments. For laboratories that lack GC-MS instrumentation, this experiment can be performed with a GC instrument alone. The identification of the various GC chromatographic peaks can be determined when compared to the retention times of standard mixtures. If an appropriate MS library is unavailable, the use of standard FAMEs can be employed to generate a library.

Due to the relatively short in-lab requirement, we couple this experiment to the isolation of bacterial DNA. This has the “economy” effect of getting two biological materials for the price of one. The DNA-isolation experiment gives rise to an analysis of a temperature-induced hyperchromicity study that demonstrates the denaturation and then reannealing of the DNA double helix. In the combined report for these two experiments, students are asked to compare and contrast the biological integrity of DNA versus the biological integrity of membranes. We think this contrast provides a wonderful demonstration of the two major weak forces that are so important in explaining many biological phenomena.

References