

**MEMBRANES, MEMBRANE TRANSPORT,
AND THE ORIGIN OF CELLULAR LIFE**

A preliminary report.

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ABSTRACT

One of the major unsolved problems related to the origin of life concerns the origin and nature of cellular membranes. All cells are bounded by a lipid bilayer that regulates the exchange of materials with their environment. It has been shown that amphiphilic molecules of abiotic origin are capable of spontaneously forming membrane-bounded compartments. What is unclear is the how and when these membrane-forming molecules became associated with self-replicating macromolecules such as RNA. RNA figures importantly in current theories of the origin of life (the RNA world hypothesis), yet it is unclear how such anionic macromolecules could mediate the transport of small molecules and ion across a lipid bilayer. The goal of this project is to better understand possible solutions to this fundamental problem in the origin of cellular life. The two specific aims of this proposal are: (1) to review the relevant literature concerning the origin of membranes, with a view toward developing testable hypotheses, and (2) to test a working hypothesis that primordial membranes may have been more porous than the membranes seen in modern cells. In order to address the latter hypothesis, protein or RNA catalysts will be encapsulated within membrane-bounded vesicles *in vitro*. These encapsulated catalysts will then be tested for their ability to yield products in the presence of polar substrates. By varying the lipid composition of the encapsulating membranes, it will be possible to identify the lipid composition of membranes that permit access of polar substrates to their encapsulated catalysts.

INTRODUCTION

The origin of cellular life is one of the most vexing problems to be faced by biologists in the next hundred years. Some essential clues have been uncovered in recent decades, most notably the discovery of the catalytic abilities of RNA molecules (Yarus, 1999). It has been argued that the dual ability of RNA to serve both as genetic material and as catalyst resolves a longstanding argument concerning the so-called chicken-and-egg problem, as to which came first, nucleic acids (present-day genetic material) or proteins (major present-day catalysts). It is hypothesized that an important step in the origin of life was the advent and evolution of the RNA world, in which RNA served as the genetic material and as the catalyst for the replication of RNA as well as a primitive form of metabolism. A careful analysis of the available evidence suggests a scenario in which RNA preceded proteins which preceded DNA.

Despite the molecular and cellular evidence for this scenario, what remains unclear is the point in these scheme at which membranes began to encapsulate these primitive self-replicating systems of macromolecules. It has been argued that membrane compartmentation of self-replicating systems of RNA molecules would facilitate the evolution of these molecules by means of natural selection, since the benefits inherent to a specific RNA molecule (or set of RNA molecules) would only accrue to those molecules within the specific membrane bounded compartment and not to other molecules present in other cells or in the prebiotic "soup". It is possible that membranes were acquired prior to the advent of RNA (since it is often argued that there may have been a simpler self-replicating polymer prior to RNA).

All cells are bounded by a lipid bilayer that regulates the exchange of materials with their environment. It has been shown that amphiphilic molecules of abiotic origin are capable of spontaneously forming membrane-bounded compartments. If it is postulated that membranes encapsulated these RNA molecules to form the earliest cells, then another important problem emerges: the problem of membrane transport in these primordial cells. In present-day cells, membrane-spanning proteins serve as carriers or channels that permit the transport of polar or charged molecules and ions. RNA would seem to be poorly suited to this purpose because of its chemical nature: the phosphodiester backbone is negatively charged and the ribose and nitrogenous bases are polar in nature. It is not clear how this macromolecule would be able to span the hydrophobic interior of a lipid bilayer, in the same way that polypeptides are able to do. On the other hand, if membrane were acquired by self-replicating systems of RNA after the origin of RNA-directed protein synthesis, another problem emerges in that selection for channel-forming

polypeptides could not take place unless membranes were already in place, which brings us back to the original difficulty. It is hypothesized that the solution to this problem is that primordial membranes were more porous and non-selectively permeable to small molecules and ions than are present-day cells, in which the transport of these molecules and ions is regulated by a set of membrane transport proteins. The importance of a diverse set of membrane transport proteins is seen in the analysis of proteins encoded by microorganisms with the simplest genomes. Even a simple microbe employs at least a few dozen membrane transport proteins to facilitate the transport of polar molecules and ions into the cell.

Another important problem to be addressed is the source of the amphiphilic molecules that comprise these primitive membranes. There are known extraterrestrial sources of these molecules, as shown by the work of Deamer and Pashley (1989), but whether these molecules were present at sufficient concentrations to form vesicles, or whether they were able to persist remains to be seen. It has been argued in recent years, that primordial organic molecules are unlikely to persist for extended periods of time, unless replenished by some mechanism. The origin of cellular lipid synthesis presents another significant problem in origin of life studies. In the modern era, lipid syntheses are carried out by complex multiprotein enzyme complexes. What is not known yet is whether RNA molecules could have promoted the synthesis of lipids, or whether lipid synthesis in cells were initiated only after the advent of proteins. Some clues to this problem may be provided by the association of intermediates in lipid synthesis with coenzymes that are ribonucleotide derivatives.

The goal of this project is to better understand possible solutions to this fundamental problem in the origin of cellular life. We propose to carry out two primary activities that are intended to point the way to making progress toward this goal. The specific aims of this proposal are:

- 1) To review the relevant literature concerning the origin of amphiphilic substances, membranes, and membrane transport in primordial cells, with a view toward developing experimentally testable hypotheses.
- 2) To address a working hypothesis that primordial membranes may have been more porous than the membranes seen in modern cells, and were selectively permeable to polar molecules (small molecules and ions permeated membrane, while macromolecules were retained within membrane-bounded compartment).

EXPERIMENTAL STRATEGY

General Strategy

In order to test the above hypothesis, protein (and later RNA) catalysts will be encapsulated within membrane-bounded vesicles *in vitro*. A considerable literature exists that describes simple methods to bring out this encapsulation, especially since these sorts of techniques are employed in the preparation of liposomes than encapsulate drugs and proteins that are to be delivered to the cell interior through the fusion of liposomes with the plasma membrane of cells. For the initial experiments, well-characterized hydrolytic enzymes have been employed due to the ready availability of chromogenic substrates. The membrane-encapsulated catalysts will then be purified by means of gel filtration chromatography using chromatographic media with a very high molecular weight exclusion limit (to ensure the separation of enzyme complexes from the membrane vesicles). The encapsulated catalysts will then be tested for their ability to yield colored products when mixed with presence of chromogenic substrates. By varying the lipid composition of the encapsulating membrane, it will be possible to identify the lipid compositions that favor the formation of membrane defects that facilitate the transport of the polar substrates across the lipid bilayer. Throughout the experiments, it will be essential to carry out a number of different types of control experiments to determine the efficiency and stability of the encapsulation process, the activity and

stability of the catalyst, as well as the extent to which the chromogenic substrate has undergone nonenzymatic hydrolysis.

Specific Goals: Preliminary Experiments and Methods Development.

1. Beta-galactosidase assays. The activity of this well-characterized enzyme (the product of the lacZ gene from *E. coli*) can be assayed by means of a simple procedure in which a colorless substrate, ortho-nitrophenyl-galactoside (ONPG) in phosphate buffer is broken down by the enzyme into ortho-nitrophenol (ONP, with an intense yellow color) and D-galactose (colorless). This chromogenic substrate generates a product ONP, the concentration of which can be measured by means of a simple visible wavelength spectrophotometer.

- a) Students will be trained to carry out this assay using a mutant strain of *E. coli* cells that produces this enzyme in a constitutive fashion (lacI⁻ cells, grown in minimal media). These cells are permeabilized by treatment with detergent (Sarkosyl) to create a cell suspension in which beta-galactosidase is accessible and abundant. This relatively inexpensive source of enzyme will be used to gain familiarity with this basic assay as well as the basic principles of enzyme kinetics used to analyze the resulting data.
- b) As soon as the students gain facility with this assay, then we will begin to employ a highly purified preparation of the beta-galactosidase enzyme (purchased from Sigma Chemical Co.). As this material is relatively expensive, it is prudent to work out the basic methods with the renewable cellular source prior to the use of the purified enzyme in subsequent experiments.
- c) In order to conserve materials, it will also be necessary to minimize the reaction volumes used. It is reasonable that the volumes used in the standard assay (3.0 ml) can be reduced 10-fold, especially through the use of special cuvettes with small volume chambers (0.2 ml).

2. Enzyme encapsulation. In the next phase of this experiment, the enzyme will be encapsulated in membrane-bounded vesicles, by using the simple method described by Deamer and Barchfeld (1982). Briefly, phospholipids (and other lipids) at 1 mg/ml are dispersed by sonication in distilled water to form liposomes. These liposomes are then mixed with the material to be encapsulated, and then small volumes of the mixture (0.2 ml) are dried under a stream of air. The dried materials are then rehydrated by sealing the tubes with a cotton plug for 2 hrs., followed by the addition of distilled water to restore the original volume of 0.2 ml. As a result of this procedure, cell-sized vesicles are formed that can be easily observed by phase contrast light microscopy (Figure 1). The vesicles created in this manner are observed to vary greatly in size, shape, and aggregation.

- a) Initial experiments have been carried out with bovine serum albumin (BSA), a relatively inexpensive, highly purified protein. These experiments are also being conducted in order to quantitate the efficiency of encapsulation. A simple pelleting procedure takes advantage of the observation that these cell-sized vesicles readily are readily pelleted by low speed centrifugation. SDS-polyacrylamide gel electrophoresis has been used to estimate the amount of protein encapsulated within the vesicles (Figure 2).
- b) It is still necessary to test the stability of the beta-galactosidase enzyme to conditions used in the encapsulation procedure. It may be necessary to add stabilizing substances to preserve the activity of the enzyme.
- c) We will also test how the efficiency of encapsulation is affected by the lipid composition of the generated vesicles. We expect to see differences based on the chemical nature of the polar head groups for the phospholipids used (positively charged, negatively charged, or neutral, dependent on the phospholipids used).

- d) In order to definitively establish the encapsulation of the proteins, we will examine the resistance of putative encapsulated enzyme to externally-added proteolytic enzymes. Encapsulated proteins should be completely resistant to proteolysis under this treatment.

3. Purification of membrane-encapsulated enzymes. The next step will be to purify the membrane-encapsulated enzyme from the remaining unencapsulated enzyme.

- a) One simple means of doing this is by pelleting the vesicles (containing encapsulated protein) through low speed centrifugation, followed by resuspension and re-pelleting to wash away unencapsulated protein. Initial experiments suggest that this may be a viable strategy, but it remains to be seen whether the manipulations involved in washing the vesicles will rupture and release the encapsulated protein.
- b) A more gentle procedure (gel filtration chromatography) will also be employed, consistent with the methods used by others, such as Deamer and Barchfeld (1982). In order to separate beta-galactosidase (about 464 kDa) from the vesicles it will be necessary to use a gel filtration chromatographic medium capable of fractionating large macromolecular assemblies (Sephacryl S-1000 SF, useful for the fractionation of macromolecular assemblies and particles between 1 to 100 megadaltons).

4. Permeability tests of membrane vesicles with varying lipid compositions. The purified vesicles, containing the encapsulated enzyme, will then be tested for their permeability with respect to the polar, chromogenic substrate of encapsulated enzyme. The amount of product generated should depend on the relative access of externally applied substrate the enzyme enclosed within the vesicles.

- a) We will systematically vary the lipid composition of the generated vesicles. We will initially examine the properties of vesicles comprised of a single lipid, followed by experiments in which the relative proportions of two or more lipids will be varied systematically.
- b) We are working on an apparatus that will make it possible to encapsulate enzyme in vesicles with up to 24 or 96 different lipid compositions, by using microtiter plates with lids modified to direct a stream of air into each of the individual wells.
- c) We also intend to test other parameters that could potentially affect the permeability of the vesicular membranes to polar substrates, including temperature, osmotic pressure, strong electric fields, and ultraviolet radiation.

PROGRESS AND PROSPECTS

During the last year we have worked to implement and establish each of the methods described above. The beta-galactosidase enzyme assay has been successfully implemented in our laboratory. The key to the success of these experiments will be the ability to encapsulate enzyme within membrane vesicles, and preliminary experiments suggest that the method described by Deamer and Barchfeld (1982) is working as intended. At present we are about ready to proceed to the step of purifying the membrane-encapsulated enzyme in preparation for the first experimental studies of membrane permeability as a function of lipid composition.

Despite the fact that the literature concerning the origin of membrane transport in early cells is sparse, there have been a number of key publications concerning this topic in the last year concerning this topic, indicating a renewed interest in a key unsolved problem concerning the origin of cellular life. To this end, an excerpt from a recent review in *Science* by Walter et al. (2000) is notable:

“For a collection of RNA molecules to have evolved from self-replicating catalysts toward systems of higher complexity, they must have become encapsulated in membranes. Only then could beneficial catalysts be selected for (because, by being sequestered in membrane-bound compartments, these enzymes were assured of benefiting from the products of the reactions they catalyzed). Thus, membranes that surrounded (and hence defined) the first cells would have been an essential feature of the earliest steps in the evolution of life. Membranes, however, pose new problems--lipids self-assemble into bilayers that spontaneously form tight barriers and, therefore, even the most primitive cells must modulate membrane permeability to permit uptake of nutrients and secretion of waste products. But RNA, because it is very hydrophilic, seems entirely unsuited for this purpose (although, surprisingly, RNA molecules that increase ion permeability in vitro have recently been identified) [Khvorova et al., 1999]. One can surmise that the need to invent proteins was strongly driven by the need to build defined hydrophobic structures that when integrated into membranes could confer selective permeability.”

This explicit statement of the very problem being considered in this study, as well as the mention of the very surprising result obtained by Khvorova et al. (1999), suggests that this unsolved problem has begun to engage the attention of the research community. As such, the prospects for procuring additional grant support for this line of investigation would seem to be favorable. Funding opportunities are therefore currently being investigated, especially through announced NASA and NSF programs.

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FIGURES AND LEGENDS

Fig. 1. Phase contrast microscopic image of cell-sized membrane vesicles (arrows) formed from phosphatidylcholine using the method described by Deamer and Barchfeld (1982).

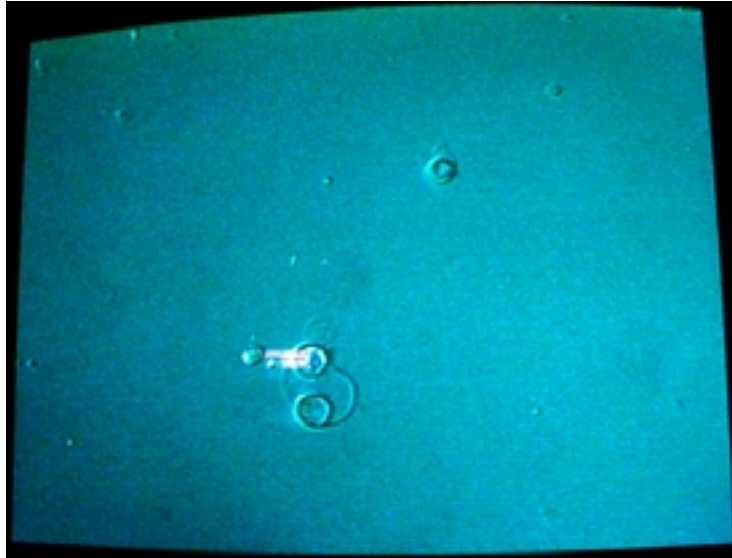


Fig. 2. SDS-polyacrylamide gel electrophoresis of pelleting experiments with bovine serum albumin (BSA) encapsulated within vesicles composed of phosphatidylcholine (PC). Lane M: Molecular weight markers. Lane T: Total mixture of PC vesicles containing BSA. Lane S: Supernatant resulting from low speed centrifugation (10,000 x g) of sample loaded in lane T. Lane P: Pellet resulting from low speed centrifugation. The samples loaded in lanes S and P were prepared to be at the same relative concentration as the sample in lane T. The small amount of protein in lane P appears to persist even when the vesicles are pelleted, washed by resuspension, and then re-pelleted.

