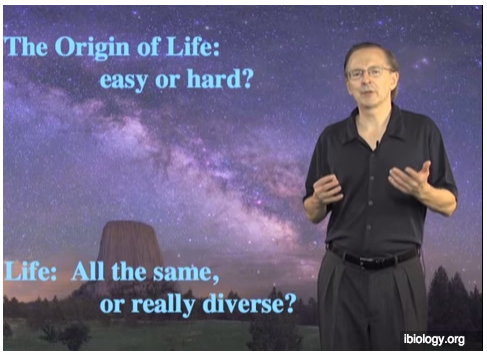
Jack Szostak
Lecture Part 1: The Origins of Life
Teaching tools prepared by Kelsey Hass
Key Words

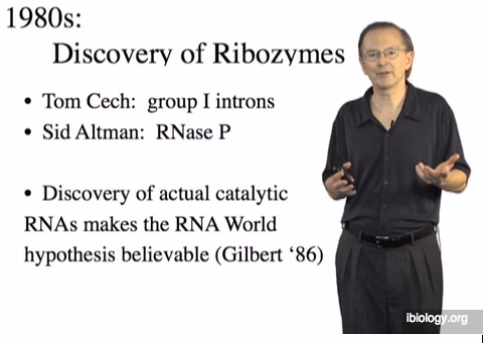
RNA world hypothesis, prebiotic chemistry, fatty acids, nucleotide triphosphates, protocell

Lecture Notes



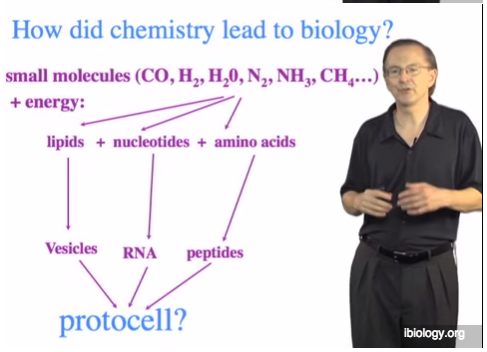
Time: 3:07

How did life originate on Earth in the harsh, extreme environments of the early planet? Was the chemistry to produce essential biomolecules relatively straightforward and simple, or was it complex and full of bottleneck steps? Is cellular life easy to evolve, or a rarity? How diverse are the biomolecular solutions that can support life? In this lecture, Jack Szostak introduces some fundamental experiments and hypotheses for the origins of life.



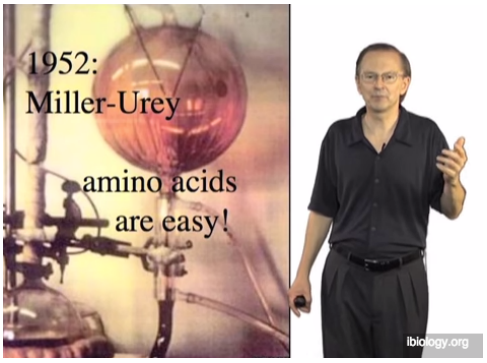
Time: 13:22

The discovery that RNA species could catalyze reactions and carry information led to the hypothesis that life emerged as a single cell carrying RNA polymer(s). As evolution continued, specialized storage molecules, transport systems, and compartments of modern cells would emerge.



Time: 21:00

How did chemistry lead to biology? One model is that simple prebiotic molecules spontaneously combined in different ways with energy input from extreme environmental conditions. The products of these combinations were primitive precursors that could further combine to generate fundamental biomolecules.



Time: 27:56

An important proof-of-principle experiment was the Miller-Urey experiment. Here, exposing reducing gases to an electric spark discharge generated many different amino acids. This suggested other biomolecules may be generated from high energy prebiotic building blocks under extreme environmental conditions.

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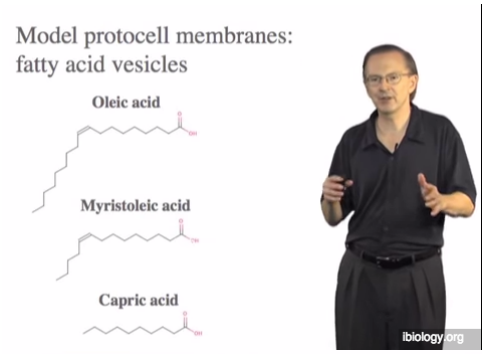
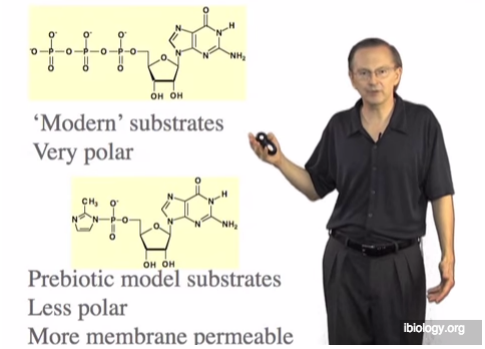


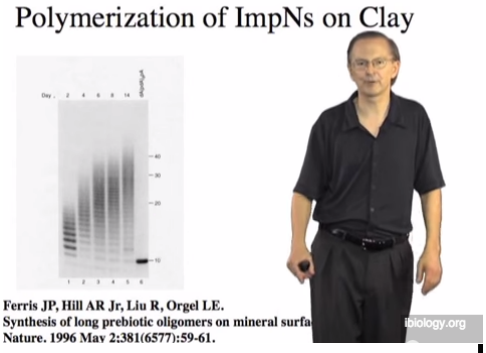
Time: 33:42

Short chain fatty acids are plausible molecules to form protocell membranes. Fatty acid membranes are permeable and allow diffusion of prebiotic molecules. Simply shaking these lipids in water, salt and buffer can form a heterogeneous mixture of different-sized single- or multi-lamellar vesicles.



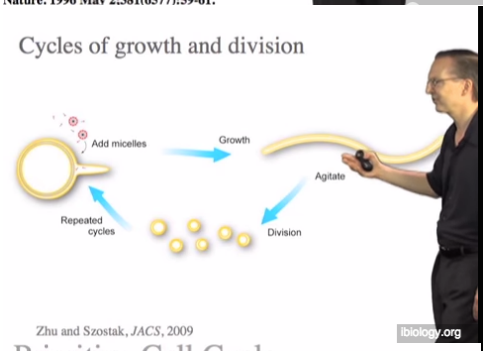
Time: 38:22

Prebiotic nucleotide triphosphates have different chemical and structural properties compared to modern nucleotide triphosphates, as summarized on this slide.



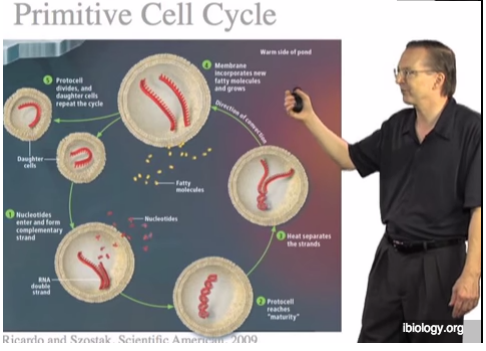
Time: 41:59

How could the first genetic material be synthesized in early Earth environments? This example experiment showed that specific prebiotic nucleotides (ImpNs) could spontaneously polymerize to form long-chain polymers when exposed to a clay surface.



Time: 50:23

The current model for growth and division of protocell membranes is summarized to the left. Vesicles can grow in the presence of fatty acid micelle food, forming filamentous structures that shear into daughter cells.



Time: 51:17

The primitive cell cycle model is summarized to the left. Here, a protocell contains genetic material molecules that can spontaneously self-replicate with rapid influx of precursor nucleotides. Genetic material can be passed down to daughter cells as the membrane grows in size and splits. The ideal environment for replicating protocells would be in geothermal convection currents; cold temperatures favor replication chemistry while high temperatures favor strand separation and division.

Jack Szostak
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Review Questions

1. What is the RNA world hypothesis?

The RNA world hypothesis states that RNA may have been the primitive cell's genetic molecule, since RNA can store genetic information in a nucleotide base code and catalyze reactions ranging from RNA self-replication to chemical/ribozyme functions.

2. What biological building block was spontaneously generated in the Miller-Urey experiment?
    a. Nucleic acids
    b. Fatty acids
    c. Amino acids
    d. Sugars
    e. Proteins

Answer: c.

3. What biomolecule is hypothesized to compose primitive protocell membranes?

Short chain fatty acids are hypothesized to compose the protocell membrane.

4. What are the chemical property/properties of prebiotic nucleotides?
    a. Less polar
    b. High membrane permeability
    c. Reactive nucleophilic group
    d. A and B only
    e. A-C

Answer: e.

5. What primitive environmental material is hypothesized to deliver RNA into vesicles and allow RNA self-replication?

Clay is hypothesized to deliver RNA into vesicles.

Discussion Questions

1. What insights from the modern world/modern ecosystems lead us to believe that life could emerge even in harsh, extreme environments?

A variety of harsh, extreme environments support microbial life. For example, microbes have been identified in hydrothermal deep sea vents (with high temperature and pressure) and Yellowstone hot springs, photosynthetic cyanobacteria have been found in acidic rocks (pH 1), and microbes have been found in acidic water bodies in Spain (pH 1.7-2.5). It is therefore plausible that early life could have emerged from similar extreme environments.

2. What experimental evidence or results might indicate that life is "easy" to emerge in harsh environments? What evidence or results might indicate that life is "hard" to emerge in harsh environments?

Early life is hypothesized to have "simple" chemical reactions that support protocell functions (eg growth, division, genetic information replication). Using modern biomolecules, we can deduce the likely prebiotic starting materials that would have been abundant in prebiotic environments. Whether life was "easy" or "hard" to emerge depends on the specific chemistry required to generate important biomolecules from these precursors. If the chemical pathways are relatively straightforward and rapid, and precursors can

undergo simple chemical reactions in the lab to generate larger biomolecules, then life can be seen as "easy" to emerge. If the chemical pathways rarely occur or contain bottleneck chemical steps (steps that limit the rate or quantity of biomolecule synthesis), then life can be seen as "hard" to emerge.

3. Explain the protocell. How could these cells undergo Darwinian evolution?

The protocell is a model primitive cell that contains a fatty acid membrane enclosing a few molecules of RNA (or other long-chain nucleic acid derivative). Vesicle membranes likely contained short chain fatty acids, as these lipids increase membrane permeability. The protocell is hypothesized to grow by engulfing fatty acids from the environment, morphing into a filamentous structure and breaking apart into new tiny protocell vesicles. Long-chain nucleic acids were likely spontaneously generated and replicated following Watson-Crick base-pairing rules on clay, ice crystals or other surfaces. Long-chain nucleic acids are composed of prebiotic nucleotide triphosphate derivatives that diffused through the protocell membrane from the environment.

After spontaneous generation, these cells could undergo Darwinian evolution because each protocell isolated specific RNA molecules inside its membrane compartment. Any additional mutation made in the RNA that conferred an advantage would only affect that protocell. This lucky protocell would then have a selective advantage for its environment, allowing it to replicate more efficiently and increase its descendants in the population.

Explain/Teach these Concepts to a Friend

1. Teach the primitive cell cycle to a friend. Explain/draw a protocell (including its membrane composition and vesicle content), and describe/draw how it is hypothesized to self-replicate.

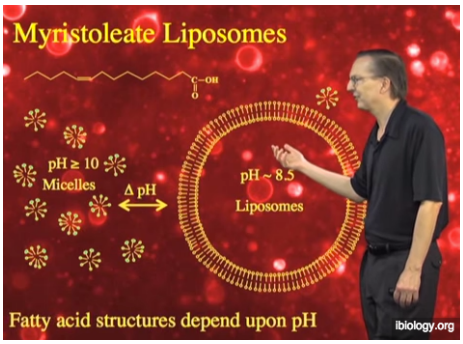
2. If you were a scientist in this field, what scientific question about the origins of life and/or protocell life cycle model would you tackle? What experiments would you conduct?
   Note: This may require students to do a bit of literature research. One advised starting point: look up Jack Szostak on PubMed, and read other papers cited in research or review articles written by members of his lab. Students should be encouraged to choose questions/experiments not yet conducted in published research articles.

Jack Szostak
Lecture Part 2: Protocell Membranes
Teaching tools prepared by Kelsey Hass

Key Words

Origins of life, protocell, fatty acids, micelle, multilamellar vesicle

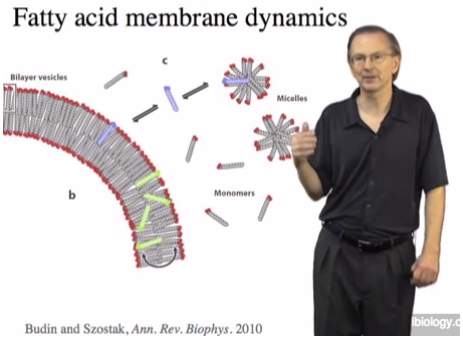
Lecture Notes



Time: 2:53

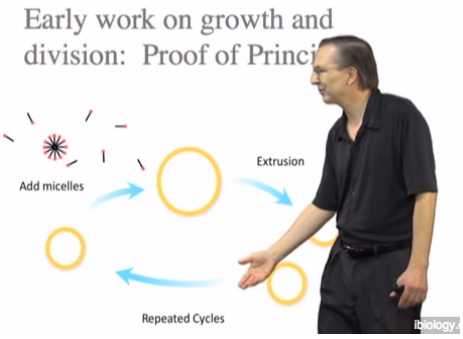
In this lecture, Jack Szostak describes characteristics of protocell membranes and experiments that support models for protocell membrane growth and division.

Fatty acid vesicle structure depends on pH. Intermediate pH favors spontaneous formation of a bilayer membrane. High pH favors formation of single-layer micelles, while low pH protonates the carboxylic acid groups of the fatty acid heads, collapsing vesicles into oil droplets.



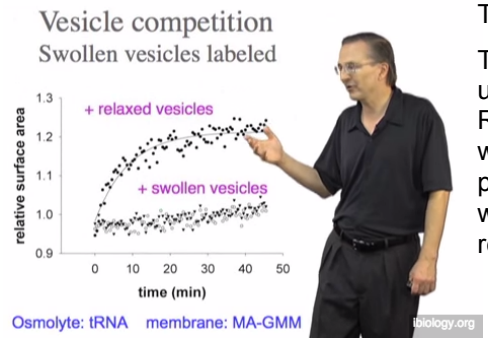
Time: 5:35

Fatty acid membranes are highly dynamic; fatty acids can exchange with the environment and within the bilayer leaflets on a time scale of seconds.



Time: 9:12

Fluorescence-based assays with donor and acceptor fluorophores anchored in vesicle membranes provided evidence that vesicles can grow in size (increase surface area) upon addition of micelle food.



Time: 14:56

The same fluorescence-based assays described above were used to investigate vesicle competition. Swollen vesicles (many RNA molecules; high osmotic pressure) were added in solution with relaxed vesicles (few RNA molecules; low osmotic pressure). Surface area for the swollen vesicles increased, while surface area for relaxed vesicles decreased. Genomic replication may therefore impact vesicle growth and replication.

Jack Szostak
Lecture Part 2: Protocell Membranes
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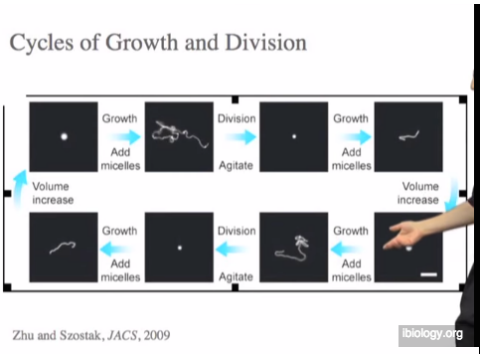
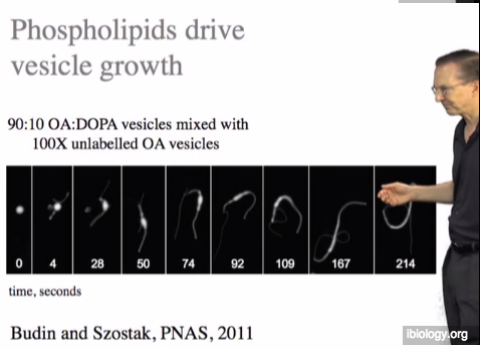


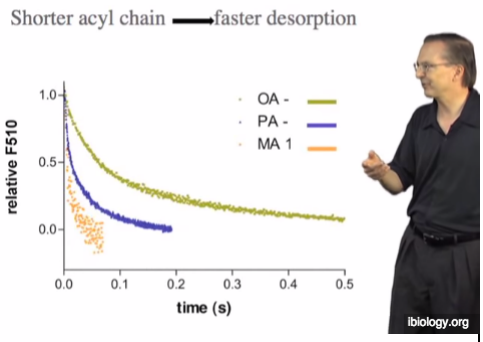
Time: 22:57

To investigate a model for protocell growth and division cycles, vesicles with a fluorescent dye encapsulated inside were visualized by microscopy. Upon addition of fatty acid micelle food, vesicles expanded much faster in surface area than volume, producing long filamentous structures. Gentle agitation or other shearing forces divided the filamentous structures into small vesicles.



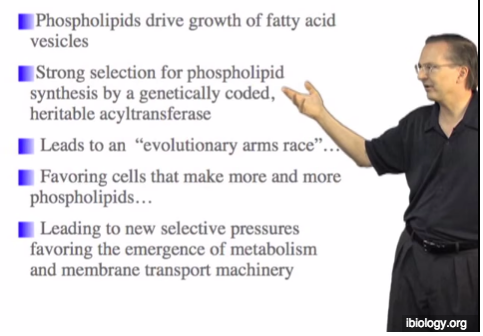
Time: 31:31

To investigate the advantage of phospholipid membranes, vesicles with 10% phospholipid composition and a fluorescent dye encapsulated inside were visualized by microscopy. Upon addition of pure fatty acid vesicles, the 10% phospholipid vesicles increased in size. Phospholipid-containing vesicles essentially “eat” their pure fatty acid vesicle neighbors.



Time: 34:50

Phospholipids contribute to membrane stability as well. The dissociation rate of fatty acids in 10% phospholipid-content membranes is slower than that in pure fatty acid membranes.



Time: 36:25

The model for phospholipid membrane evolution is summarized to the left.

Phospholipid incorporation drives vesicle growth, which in turn creates a selective pressure for RNA that encodes phospholipid synthesis enzymes. As evolution continues, cells that contain higher phospholipid composition have a greater advantage for survival. Since phospholipids are less permeable membranes, new selective pressures drive evolution for membrane transport mechanisms.

Jack Szostak
Lecture Part 2: Protocell Membranes
Teaching tools prepared by Kelsey Hass

Review Questions

1. What are the two main properties of fatty acid dynamics in protocell membranes?

The two main properties are: (1) fatty acids interexchange between the two membrane layers and between the outer layer and the environment, and (2) these exchanges or "flip-flops" occur on the second time scale.

2. What real-time fluorescence-based assay (used for many of the experiments shown in this lecture) allowed researchers to test protocell membrane properties and dynamics?

The fluorescence-based assay is called FRET (fluorescence resonance energy transfer), which relies on fluorescence energy transferred from donor dyes to acceptor dyes anchored in the membrane. [*When the donor and acceptor dyes are close in distance, fluorescence energy transfer from donor fluorophore dye to acceptor fluorophore dye is very efficient. This produces a strong fluorescent signal by the acceptor dye. Conversely, when the donor and acceptor dyes are far apart in distance, fluorescence transfer from donor to acceptor dye is very inefficient.]* Here, donor and acceptor dyes were anchored into vesicle membranes to measure vesicle growth and division in real-time. When vesicles grow by adding new molecules to their membrane, acceptor and donor dyes are diluted further apart, and fluorescence transfer efficiency decreases. This results in a weak fluorescent signal output from the acceptor dye.

Note: The italicized comments are extra information not included in the video that may be helpful in explaining this assay to students.

3. What is the intermediate structure of replicating vesicles?
    a. A budding capsule
    b. Long, filamentous structures
    c. Spiral-shaped vesicular tubes
    d. Vesicles haven't yet been shown to self-replicate

Answer: b.

4. Choose the four correct properties describing how phase transitions can form fatty acid vesicles.
    a. Low pH
    b. High pH
    c. Intermediate pH
    d. Hydrogen bonding between adjacent amino groups of fatty acids
    e. Hydrogen bonding between adjacent carboxyl groups of fatty acids
    f. Ionic bonding between adjacent head groups of fatty acids
    g. Repulsion forces between adjacent head groups of fatty acids
    h. Repulsion forces between hydrocarbon tails of fatty acids
    i. Aggregation forces between hydrocarbon tails of fatty acids

Answer: c, e, g, and i

5. Fill in the blanks: _____ grow in size when micelle "food" is added to form _____ that divide or break apart when exposed to environmental stresses such as _____ stress.

Answer: Fatty acid vesicles ("*protocells" or "vesicles" also acceptable)*; filamentous structures; mechanical (*"wave action" or "agitation" also acceptable)*

6. True or false: Phospholipids were typical membrane components for early life protocells.

Answer: False.

Jack Szostak
Lecture Part 2: Protocell Membranes
Teaching tools prepared by Kelsey Hass

Discussion Questions

1. What are some characteristics of a protocell membrane? How do these differ from a modern cell membrane?

Protocell membranes are much more permeable than modern cell membranes, because diffusion of molecules (nucleotides, ions, etc) was the primary transport mechanism into the cell. As such, protocell membranes are hypothesized to contain short chain fatty acids, like capric acid, mysteraic acid and glycerol esters. The protocell "leaky membrane" is not characteristic of modern cell membranes, which are much more rigid and impermeable to small molecules. The increase in impermeability is reflected in modern cell membrane composition of primarily phospholipids.

2. What is the current model for protocell growth and division? Describe the experiments that support this model.

Multi-lamellar (multiple-membrane) protocells grow by absorbing fatty acid foods, such as micelles or neighboring fatty acid vesicles with lower osmotic pressure. Vesicles then form long, fragile filamentous structures. Mechanical stress, gentle agitation or shearing forces then break apart the filamentous structure into small new vesicles. The growth/division cycle then repeats.

A combination of experimental approaches helped define this model. Fluorescence-based assays with donor and acceptor dyes anchored in vesicle membranes showed that vesicles absorb surrounding micelle or fatty acid foods. As the membranes grew bigger, the donor and acceptor dyes increased in distance, and fluorescence transfer efficiency decreased. Assays using a fluorescent dye encapsulated inside a fatty acid vesicle were used to visualize vesicle size under a microscope. When food was added, long filamentous vesicle structures were visible. Thus, researchers were able to identify the intermediate structure of the vesicle growth and division cycle.

3. What is the proposed link between genome replication and membrane growth? Describe the experiments that support this model.

The proposed link is that faster genome replication induces faster membrane growth. Vesicles with encapsulated RNA molecules generate an internal osmotic pressure, because ions (and therefore water) travel inside the vesicles to counteract the charge of the nucleic acid polymer. Vesicles with more encapsulated RNA will have a higher osmotic pressure. This drives competitive growth, as vesicles with high osmotic pressure "eat" neighboring fatty acid vesicles with lower osmotic pressure.

This model is supported by experiments that combined swollen vesicles (high osmotic pressure; large numbers of encapsulated RNA molecules) with relaxed vesicles (low osmotic pressure; little encapsulated RNA molecules). When fluorescent donor and acceptor molecules were placed in the swollen vesicle membrane, fluorescence transfer efficiency decreased and thus surface area increased. The opposite is true when fluorescent donor and acceptor molecules were placed in relaxed vesicle membranes; surface area decreased for relaxed vesicles. This implies fatty acids from the relaxed vesicles were transferred into the swollen vesicles. Therefore, increased amounts of genetic material inside a protocell drive vesicle growth.

4. What proposed evolutionary pressure drove the incorporation of phospholipids into protocell membranes? Describe experiments that support this model.

Phospholipids in the membrane of a vesicle are hypothesized to provide a "protective" advantage against neighboring fatty acid protocells absorbing contents of its membrane. Phospholipids change the properties of a bilayer membrane; the dissociation rate of fatty acids from the membrane is slowed when

phospholipids are present. Therefore, a protocell with phospholipids in its bilayer has a decreased chance of being “eaten” by its neighbors.

The experiments that support this model combined vesicles with 10% phospholipid membrane composition and vesicles with pure fatty acid membrane composition. The phospholipid-containing vesicles encapsulated a fluorescent dye so that vesicle size could be visualized over time. From this assay, researchers observed that the surface area of the phospholipid-containing vesicles grew over time. An additional real-time assay measured the dissociation rate of fatty acids from phospholipid-containing vesicles. Using a pH-sensitive reporter dye inside of phospholipid-containing vesicles, the Hamilton desorption rate assay showed that fatty acids were lost from the phospholipid-containing membrane at a slower rate.

Explain/Teach these Concepts to a Friend

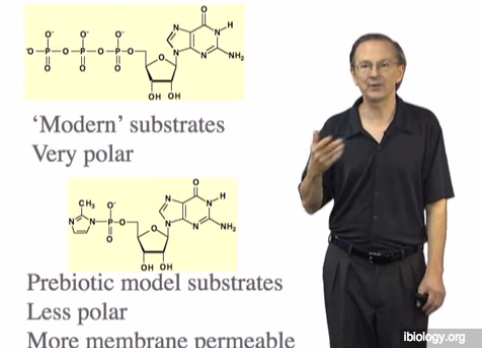
1. Explain/draw how the FRET fluorescence-based assays allowed researchers to visualize vesicle growth and division in real-time. What is the input fluorescence wavelength? Why does the distance between donor and acceptor dyes matter? What is the output fluorescence wavelength?
   Helpful hints: Students should start by drawing a small vesicle. Ask students where the donor and acceptor fluorophore dyes would be located. What would the FRET result look like on a small vesicle? Now draw a bigger vesicle. What happened to the distance between donor and acceptor dyes? How is this reflected in the assay?

2. Recall the “Donnan effect” from this lecture. Teach a friend how this hypothesis links vesicle membrane dynamics with other aspects of the protocell membrane cycle discussed in Lecture 1. It would be helpful to draw out the protocell membrane life cycle for your friend.

Jack Szostak
Lecture Part 3: Non-enzymatic Copying of Nucleic Acid Templates
Teaching tools prepared by Kelsey Hass
Key Words

RNA world hypothesis, prebiotic chemistry, protocell, imidazole nucleobase, self-polymerization

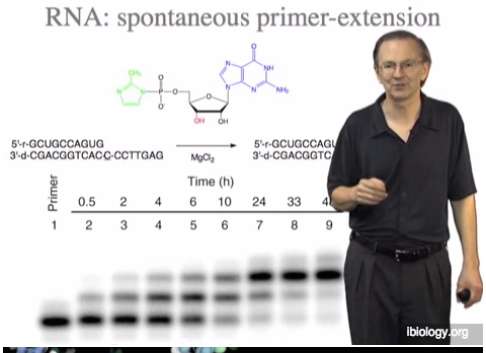
Lecture Notes



Time: 4:35

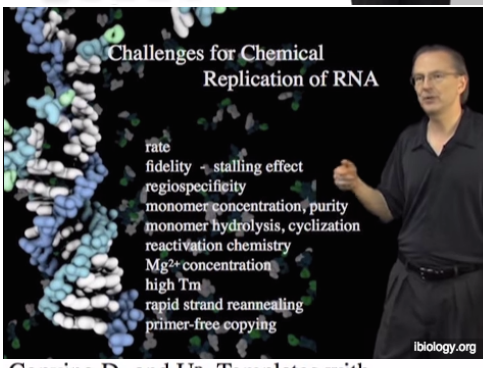
In this lecture, Jack Szostak describes characteristics of prebiotic nucleic acids, and experiments that support models for non-enzymatic replication.

Prebiotic nucleotide triphosphates have different chemical and structural properties compared to modern nucleotide triphosphates.



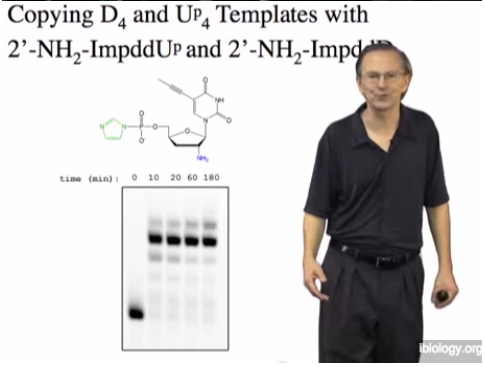
Time: 13:59

Imidazole derivatives of nucleotide triphosphates can undergo spontaneous chemical replication of RNA templates. It is an intrinsically slow process; the experiment to the left shows that synthesizing two bases can take about 24 hours. An additional problem is that the rates of synthesis and degradation are on similar time scales when the reaction proceeds in high concentrations of magnesium ions.



Time: 16:12

There are many additional challenges for chemical replication of RNA, summarized to the left.



Time: 29:22

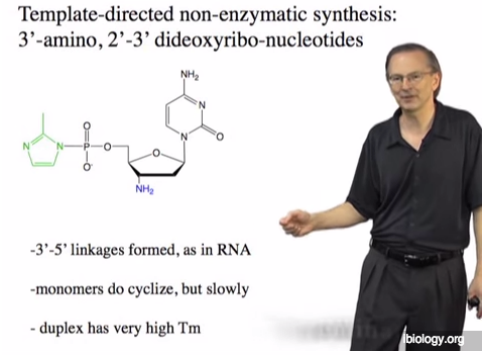
To solve the fidelity problem, one area of research is investigating alternative nucleotide derivatives. There are many chemical additions or modifications that have been shown experimentally to increase the speed and/or fidelity of spontaneous RNA template copying.

Here, amino-uracil derivatives are shown to rapidly polymerize RNA primers.

Jack Szostak

Lecture Part 3: Non-enzymatic Copying of Nucleic Acid Templates

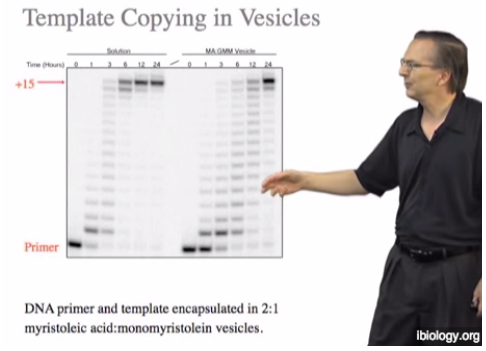
Teaching tools prepared by Kelsey Hass



Time: 34:56

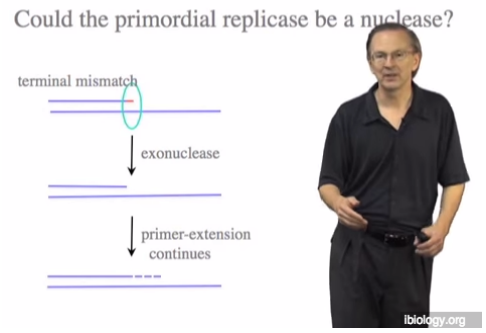
To solve the fidelity problem, one area of research is investigating alternative nucleotide derivatives. There are many chemical additions or modifications that have been shown experimentally to increase the speed and/or fidelity of spontaneous RNA template copying.

Here, imidazole-nucleotide derivatives are promising precursors for synthesizing growing RNA chains.



Time: 40:58

Prebiotic nucleotide precursors can diffuse across fatty acid membranes to form replicated RNA products, indicating the self-replication chemistry is compatible inside vesicles. One major area of research remaining is to combine the replication chemistry with growing/dividing vesicles to watch early life emerge.



Time: 50:20

Why could the primordial replicase be a nuclease? One hypothesis is that a nuclease could alleviate the fidelity stalling effect when an incorrect base is incorporated. If the primordial replicase was a nuclease, the incorrect base could be excised to maintain fidelity and the rate of synthesis.

Review Questions

1. Fill in the blanks: Prebiotic nucleotides are less _____ and more _____ than modern nucleotides.

Answer: polar; membrane permeable

2. List two plausible natural environments that could facilitate generating RNA chains.

Polymerization of prebiotic nucleotides on clay and ice eutectic phase could facilitate generating RNA chains.

3. What bond formation was considered a major roadblock for prebiotic pyrimidine synthesis?

The glycosidic bond (or linkage) in pyrimidine nucleosides serves as a roadblock for pyrimidine synthesis; it cannot be formed from hypothesized prebiotic precursors. A different pathway using an alternative intermediate molecule was needed to generate pyrimidine nucleosides.

Jack Szostak
Lecture Part 3: Non-enzymatic Copying of Nucleic Acid Templates
Teaching tools prepared by Kelsey Hass

4. What are two problems with the high magnesium concentrations needed for spontaneous RNA polymerization? Select all that apply.
    a. Magnesium is used for synthesis of RNA, but also for degradation of RNA.
    b. Magnesium ions were likely not found in prebiotic environments.
    c. Magnesium ions need to be coordinated to another metal for RNA polymerization.
    d. Magnesium can disrupt fatty acid vesicle membranes, causing fatty acid precipitation.

Answer: a and d.

5. True or false: Given the right membrane permeability, prebiotic nucleotides could flow across a protocell membrane to be accessed for RNA self-replication.

Answer: True.

6. What could be the primordial replicase?
    a. RNA polymerase
    b. DNA polymerase
    c. Nuclease
    d. Ribozyme

Answer: c.

Discussion Questions

1. What is structurally and catalytically different about the substrates required for chemistry-driven RNA/DNA replication? How could they have been generated on early Earth?

The substrates for non-enzymatic chemistry-driven RNA/DNA replication are more reactive nucleophiles. This means the substrate contains a more reactive group (such as an imidazole group) that will attack the RNA/DNA backbone to spontaneously add another base to the growing RNA/DNA strand. In addition, these substrates are less polar and can more easily or rapidly diffuse across the protocell membrane.

Hypotheses for generating nucleotides on early Earth include: self-assembly of ribose from five formaldehyde units; adenine from five cyanide units (pentamer of cyanide); and cytosine from cyanoacetyline and urea units. These simple prebiotic units can be spontaneously combined together into bigger macromolecular structures by UV light, intense heat/boiling, and other extreme environmental processes.

2. Describe some of the challenges for chemical replication of protocell genetic material. What evidence strongly suggests chemical replication is possible?

There are many challenges for chemical replication of protocell genetic material (*students may have a variety of answers, including)*:

- No direct pathway established (yet) for purine synthesis (still an active topic of research)
- Unknown how to generate pure, concentrated pools of starting nucleotide monomers from spontaneous combinations of precursor units
- In high concentrations of magnesium ions, the rate of RNA synthesis and rate of RNA degradation are on the same time scale (synthesis possible, but not sustainable for the cell to replicate its genetic material)
- Slow synthesis rate
- Stalling effect for polymerizing a correct base after an incorrect base is added
- Lack of regiospecificity (2' or 3' OH linkages can form spontaneously)
- Activated monomers are unstable; monomers can hydrolyze or cyclize, and no longer be useful for long chain generation

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Teaching tools prepared by Kelsey Hass

- High melting temperature of RNA template and copied strand duplex; strands are hard to dissociate for further template copying
- Rapid RNA strand re-annealing (same problem as above)
- GC base pairing works well in current research experiments, but AU base pairing is much slower or does not work at all

Despite these challenges, there are many example long-chain molecules in the lecture, such as GNA, TNA, and MoNA, that suggest chemical replication is possible. At the most basic level, there is a plausible pathway for pyrimidine synthesis from spontaneous combinations of prebiotic units. Simple precursors can combine to generate an intermediate that can undergo further spontaneous manipulations to become cytosine, or deaminated to become uracil. This is promising and suggests nucleotides and longer chain nucleic acids could be generated spontaneously. Polyacrylamide gel electrophoresis results showed that longer chains of RNA could be spontaneously synthesized over time from variations of different starting nucleotides (all with different nucleophile groups or slightly different chemical structures and reactivity). The prominent example in this lecture is 2'-amino, 2',3'-dideoxyribonucleotides (2'-NH_2 ImpdG).

3. Give examples of current research efforts to solve the fidelity problem with RNA replication.

Examples of current research efforts include (*students may have a variety of answers)*:

- Using another base analog, 2-thiol-uracil, that has a more reactive sulfur group and promotes tighter (higher fidelity) adenine/uracil base pairing
- Synthesizing conformationally constrained RNA long-chain backbone structures (ex 3'-phosphoramidate DNA; threose nucleic acid or TNA; morpholino nucleic acid or MoNA) that may favor the incorporation of the right base simply by being constrained to favor one conformation
- Investigating the possibility that ribozymes, small molecules or peptides may catalyze long-chain formation (i.e. using a catalyst that favors specific conformations of monomers and backbone products, instead of spontaneous non-enzymatic chemistry)

4. What is the major takeaway about monomer (or nucleotide base) homogeneity?

Monomer homogeneity is specific to the lab setting; the simplest experiments to investigate scientific questions use one type of pure monomer at high concentrations. However, in "real prebiotic life", there would be a heterogeneous mixture of monomers created from all possible random, spontaneous combinations of prebiotic precursor units. Therefore, a mixture of DNA or RNA bonds could form within the same molecule from combinations of these heterogeneous monomers. In fact, as shown in the lecture, a mixture of 2'-5' and 3'-5' linkages within the same molecule reduced the melting temperature for RNA duplexes, and may have been an essential characteristic to promote strand separation for RNA self-replication.

Explain/Teach these Concepts to a Friend

1. Teach a friend why modern nucleotide triphosphates and phospholipid membranes are not suitable candidates for nucleic acid self-replication chemistry in a primitive protocell. Describe what properties of nucleotides and lipids would have to change in order for these molecules to be suitable for a prebiotic protocell.

2. Tackle one of the challenges for chemical replication of protocell genetic material mentioned in the lecture. What experiments would you perform to investigate this challenge?
   Note: This may require students to do a bit of literature research. One advised starting point: look up the articles cited in this lecture on PubMed, and read other relevant research or review articles. Students should be encouraged to choose questions/experiments not yet conducted in published research articles.

Jack Szostak
Delve Into the Literature: Non-enzymatic Template-Directed RNA Synthesis
Teaching tools prepared by Kelsey Hass
Discussion Paper
Adamala, K. and Szostak, J.W. "Nonenzymatic Template-Directed RNA Synthesis Inside Model Protocells." 2013. *Science*. 342: 1098-1100.

Discussion Questions

Note: Students are not limited to answers provided below; answers serve as one example for the discussion section. Students are encouraged to pursue other avenues of discussion for each question.

1. What was the "roadblock" in non-enzymatic RNA replication chemistry that the authors addressed?

The authors' model for prebiotic cells has two major components: fatty acid membranes and RNA molecules that self-replicate. The chemistry to accomplish RNA copying requires a high concentration of magnesium ions, among other divalent (2+ charged) ions. However, in high concentrations of magnesium, the fatty acids become water-insoluble and aggregate into clumps (precipitate out). This destroys spontaneous arrangement of fatty acids into vesicles to form the prebiotic cell. The major roadblock addressed in this paper is resolving the incompatibility between fatty acid integrity as vesicles and successful RNA copying in high concentrations of magnesium.

Other avenues of discussion:
Why do you think magnesium ions are required for RNA replication chemistry?
[Magnesium ions are necessary for catalysis chemistry in linking free-floating nucleotide triphosphates to the growing RNA strand backbone.]
If you were given this "roadblock", what experiments would you do to find a solution?

2. Talk through Figure 2. What did they test, and how? Describe the main conclusion(s). What could be some biases in their primer-extension assay? What properties of chelators are beneficial for a primitive protocell?

In Figure 2, the authors tested the rate of RNA synthesis in the presence of Mg^{2+} and/or the chelators identified in Figure 1 that protected fatty acid membranes.

The rate of RNA synthesis was tested by primer-extension assay. Here, RNA duplexes were pre-formed such that two strands, a template and primer, were stably base-paired into a duplex at room temperature. The template strand contained an additional stretch of 4 cytosine residues, and the corresponding strand (lacking the C residues) served as a primer for spontaneous base addition of primitive guanosine derivative, 2MeImpG. The primer strand also contains a Cy3-fluorophore at the 5'end, which allows the authors to visualize the length of the primer strand over time by gel analysis. At different time points, aliquots of the reaction were taken. RNA was isolated by ethanol precipitation, and analyzed by gel electrophoresis. Gel bands were visualized by fluorescence imaging. The results would be similar to the gel in Figure 2C, which shows the amount of primer extension (all bands except the bottom row) and unused primer template (bands in the bottom-most row of the gel) with 50mM Mg^{2+} and 200mM citrate over time.

Gel band intensities were then quantified by a specific analysis program. The quantitation is useful to determine total primer intensity (P_0 in Figure 2A; all bands per one lane in 2C) and unextended primer intensities (P in Figure 2A; bottom-most band per one lane in 2C). Graphing the ratio of unextended primer/total primer (y-axis Figure 2A) over time (x-axis Figure 2A) gives the rate of RNA synthesis (essentially, depletion of unextended primers over time). The slope of the graph represents the rate of RNA synthesis. The slope of the line for each condition in Figure 2A is plotted in Figure 2B.

Looking at Figures 2A and 2B, we see the 50mM Mg^{2+}/no chelator condition gives the smallest amount of unextended primer, and the maximal rate of RNA synthesis at 1.4 $hour^{-1}$. However, this concentration of Mg^{2+} is incompatible with membrane vesicles (see question 1 above or authors' supplementary text). Of

the two chelators tested with 50mM Mg^{2+}, citrate allows the highest rate of RNA synthesis at 0.67 $hour^{-1}$. The rate of RNA catalysis with Mg^{2+} and EDTA is close to 0 $hour^{-1}$, showing EDTA is too efficient of a chelator (it immobilizes too many Mg^{2+} ions thereby depleting the stock available for RNA catalysis, resulting in large amounts of unextended primer over time).

The main conclusion of Figure 2 is that the membrane-protective chelator, citrate, allows a reasonable rate of non-enzymatic RNA template copying in 50mM Mg^{2+}, both in solution and with oleate vesicles.

One potential bias of the primer-extension assay is the choice of nucleotide base. The authors measured synthesis rates with only one base, the guanosine derivative 2MeImpG. However, primitive adenine and uracil bases are known to have slower synthesis rates in non-enzymatic RNA synthesis chemistry. It is unknown if citrate could be an effective chelator for all bases (A, U, C and G), or if the timing would be too slow with other bases (A, U) for sufficient RNA synthesis in protocells.

In model protocells, chelators would perform two roles: (1) protection against fatty acid membrane dissolution by high concentrations of Mg^{2+} ions, and (2) protection against Mg^{2+}-induced RNA degradation.
Other potential answer: Chelators must prevent increases in the melting temperature (Tm) of a RNA duplex. If the melting temperature is high, the duplex is stable. Spontaneous "unwinding" of the duplex to allow single-stranded template copying will occur less frequently. Therefore, with high melting temperatures, the protocell would not be able to quickly replicate its RNA.

3. Talk through Figure 3. What did they test, and how? What is the "liposome dialyzer", and what is the method's significance for the paper (focus on 3E)? Any critiques for this figure?

In Figure 3, the authors measured the amounts of primer extension (RNA synthesis) in different membrane vesicle compositions over time.

RNA synthesis was tested by primer extension assay, with a template region of 7 C residues (Figure 3A-D) or template region of mixed G and C residues (Figure 3E). Primer strands are complementary to template strands but lack the specified template regions, promoting non-enzymatic RNA synthesis off of the 3' end of the primer. The authors formed vesicles with different fatty acid compositions (Figure 3B-D), and each vesicle encapsulated template-primer duplex RNA, Mg^{2+} ions and citrate. Vesicles were incubated with 2MeImpG nucleotide base (Figure 3A-D), or a mixture of prebiotic G- and C-derivative bases (Figure 3E). At indicated times, the authors collected aliquots of vesicles, extracted and purified the RNA, and analyzed primer RNA length by polyacrylamide gel electrophoresis. Primer extension was visualized by fluorescence imaging of the 5' Cy3 fluorophore signal on the primer. Bands in the bottom row of the gel correspond to unextended primer, and bands at increasing lengths represent one base extended (per interval) on the primer strand.

As shown in Figures 3A-D, most of the primer is extended by 24-72 hours after one-time addition of excess nucleotide base. Figure 3A serves as a control reaction (in solution, no vesicles), while Figures 3B-D show non-enzymatic RNA synthesis in three different model protocell membrane compositions. 3D shows the most prebiotically plausible membrane composition, as it consists of shorter chain lipids that are naturally permeable to reagents like prebiotic nucleotide triphosphates.

Figure 3E analyzes two important conditions: the efficiency of synthesizing across a mixed G/C-base template region, and the efficiency of RNA catalysis with fresh monomer addition. Fresh monomers are added by incubating vesicles in a liposome dialyzer over the course of the reaction. In the lipid dialyzer, vesicles are constantly exposed to fresh sources of the prebiotic nucleotide triphosphates. This is significant because the lipid dialyzer more accurately mimics the environmental conditions that prebiotic protocells would have experienced. With fresh monomers, primer extension occurs as efficiently for vesicle-encapsulated RNA primers (lanes 1 and 2, Figure 3E) as for RNA primers in solution (no vesicles, lane 3, Figure 3E). Addition of fresh nucleotide bases results in a more intense fully-extended primer

band (lane 3, Figure 3E) than one-time addition of nucleotide bases (lane 4, Figure 3E), implying fresh addition of nucleotide base can increase the amount of extended primer at a given time point.

One critique for this figure is that "mixed bases" only refers to G/C-base template. There is still no data offered on A and U base synthesis.
Other avenues of discussion: Is citrate a viable prebiotic molecule? Would it have a more likely prebiotic form (for example, the "prebiotic form" of guanosine nucleotide triphosphate is 2MeImpG), and would this prebiotic form act differently?

4. Comment on the time scale for RNA replication vs. RNA degradation in a fatty acid vesicle containing Mg^{2+} and citrate. Does this seem plausible for early life?

RNA replication with 50mM Mg^{2+} and 200mM citrate in solution or in oleate vesicles was ~0.7 $hour^{-1}$, while RNA degradation with 50mM Mg^{2+} and 200mM citrate in solution was ~0.004 $hour^{-1}$. With these numbers, spontaneous RNA synthesis occurs ~175 times faster than degradation when 2MeImpG nucleotide base is in excess. The time scale is certainly plausible for a protocell to non-enzymatically replicate its RNA.

5. Recall the model for the protocell life cycle (Jack Szostak iBio seminars). To what extent does this paper piece together critical elements of the model? What aspects of the model remain to be tested?

The model for the protocell life cycle involves spontaneous protocell vesicle membrane growth/division and spontaneous, non-enzymatic replication of vesicle-encapsulated RNA. This paper focuses on the latter, in particular resolving the incompatibility between high Mg^{2+} concentrations required for RNA replication chemistry and Mg^{2+}-catalyzed destabilization of the vesicle membrane. The Szostak lab has previously shown that vesicles can spontaneously grow and divide through a multilamellar vesicle intermediate; this paper shows RNA molecules can self-replicate in fatty acid vesicles with Mg^{2+} and a chelator. What remains to be tested is combining the two facets together: combining the replication chemistry with conditions that promote self-replicating vesicles to mimic the complete protocell life cycle.