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How to Make ATP:

Three Classic Experiments in Biology

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Introduction

There is an old joke about biochemists:

Q: How do biochemists figure out how a wristwatch works?

A: They put it in a blender, grind it up, centrifuge out the solids and see what happens in the supernatant.

Why is this funny?

The joke goes back to the idea that in the early 20th century, many biochemists thought of cells as "bags of enzymes." Biochemists elucidated many metabolic pathways during this time by studying the proteins, enzymes, and the reactions they catalyzed. They used an experimental approach where each component of the system (enzyme, substrate, cofactors) was isolated and then reconstituted to demonstrate the reactions *in vitro*, or in a test tube. This was a very powerful technique that was used to figure out most of the biochemical pathways we know today. Because this was the main experimental approach, it was often called the conventional or orthodox approach.

Biochemists think about reaction pathways. If you have a starting substrate and wind up with a reactant, there must be intermediate molecules with enzymes catalyzing each step of the pathway. For example, in glycolysis there are enzymes that catalyze each reaction, forming intermediate molecules at each step between glucose and pyruvate. The conventional experimental approach to figuring out this pathway involved breaking cells open, isolating the components essential to the reaction (enzymes, substrates, any cofactors) and then reconstituting the system.

Energy Within Cells

Energy flow is the prime characteristic of life. Living cells take energy from their environment and transform it and store it so that the energy can then be used for all cellular processes such as growth, reproduction, communication, and movement. Without ways to capture and store energy, cells could not function.

By the early 1940s, scientists knew that energy in the cell was stored in adenosine triphosphate or ATP. Making ATP is an endergonic or unfavorable reaction that requires the input of energy. In biological organisms, that energy is supplied by sunlight (for photosynthesis) or chemicals such as sugars (for respiration). Once formed, ATP can be hydrolyzed (or split) to adenosine diphosphate (ADP) and inorganic phosphate (P*i*), releasing a large amount of energy. This energy is used by the cell to drive metabolism (Figure 1).

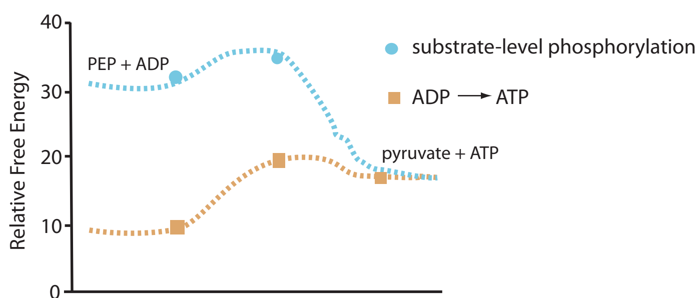

Figure 1. ATP.

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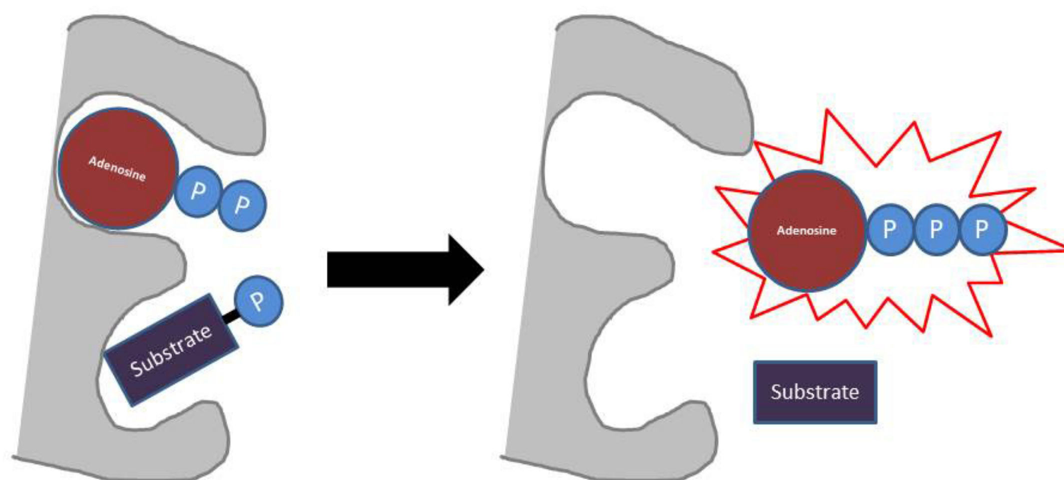

Figure 2. Substrate-level phosphorylation.

Biochemists who were studying glycolysis knew that sometimes a phosphate could be transferred to ADP via substrate-level phosphorylation, forming a high energy bond and ATP (Figure 2). It happens twice during glycolysis (when 1,3 bisphosphoglycerate is converted to 3-phosphoglycerate and when phosphoenolpyruvate is converted to pyruvate). It can occur in other metabolic reactions, for example when creatine phosphate donates a high energy phosphate to ATP in heart muscle. Substrate-level phosphorylation is an anaerobic process as oxygen is not required for it to occur.

By the 1950s, the Krebs cycle had been worked out using the conventional experimental approach, but it remained puzzling how so much ATP was formed in the presence but not absence of oxygen. Scientists knew about high-energy electron carriers (NAD and FAD), and they knew that these carriers transferred high energy electrons to a series of molecules in the inner mitochondrial membrane known as the electron transport chain (or the respiratory chain or the oxidation chain). When oxygen is present, it accepts electrons at the end of the chain, and ATP is made. Biochemists set their sights on understanding how the ATP was made in the mitochondria, and they looked for intermediates and enzymes that would catalyze the reactions. They put a significant amount of effort into trying to isolate enzymes and intermediates, trying to purify the components independent of the membranes, which they felt were contaminating their preparations. Scientists spent years trying to manage the technical difficulties they encountered attempting to isolate functional enzyme preparations, and it took more than a decade for many biochemists to be open to the possibility that the conventional approach might not work for this problem.

In 1961, British biochemist Dr. Peter Mitchell, on the faculty of the University of Edinburgh, proposed a hypothetical mechanism that coupled electron transfer to ATP synthesis. He suggested that the flow of electrons through electron carriers in membranes in either chloroplasts or mitochondria drove protons to create an electrochemical gradient. He hypothesized that this electrochemical gradient could then drive ATP synthesis through a process that was called chemiosmosis. Most biochemists were chasing elusive intermediates in the ATP pathway, and it was not easy for scientists to put together the pieces that were needed to accept the chemiosmotic theory of ATP production.

Dr. Mitchell won the 1978 Nobel Prize in Chemistry for his theory. In 1979, almost two decades after he first proposed ATP production via chemiosmosis, Mitchell reflected on his pioneering work by stating that there were three questions that needed to be answered before his 1961 hypothesis about electron transport chains in both mitochondria and chloroplasts could be accepted by the scientific community: *What are they? What do they do? And, how do they do it?*

It was the meticulous work of many biochemists who approached the challenges from different directions that led to the answers. This case study will highlight some of the key experiments and how they led to our current understanding of ATP production via chemiosmosis or a proton motive force.

Part I – What Are They?

Paper #1: Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* 191:144-148.

This paper hypothesizes the coupling of phosphorylation to electron transfer across a membrane. In the introduction to his paper, Mitchell acknowledged how different his approach to the problem of ATP synthesis was by calling the reductionist biochemical approach the "orthodox" approach at the time. He suggested that somehow the components involved in ATP synthesis needed to be spatially positioned within the membrane so that the individual elements would transfer electrons and protons (the elements of water: H^+ and OH^-) across the membrane.

What does spatial positioning mean? It means that the location of each component in relationship to the other components in the system is important if the system is to function. For example, a toy truck won't work if all of the pieces are scattered on a table. The truck only works when the pieces are assembled properly in space, as illustrated in Figure 3.

Figure 3. A metaphor to highlight the importance of spatial positioning. A truck is more than just the sum of its parts.

While it may not seem radical now, this concept was so contrary to the thinking at the time that Mitchell devoted much of his introduction to acknowledging that he was proposing a novel or unorthodox approach to the problem. He suggested a model in which the intermediates in the pathway for ATP synthesis needed to be positioned in space within a membrane, not just mixed all together in the cell cytoplasm.

Mitchell presented theoretical calculations to show how a reversible ATPase system located in a membrane could separate charges on the outside and inside of the membrane. Based on his calculations, he came up with the diagram in Figure 4.

Questions

1. Based on the diagram, is the overall charge outside the membrane positive or negative?
2. Based on the diagram, is the overall charge inside the membrane positive or negative?
3. Which side of the membrane has a lower pH?
4. Which side of the membrane is more acidic?

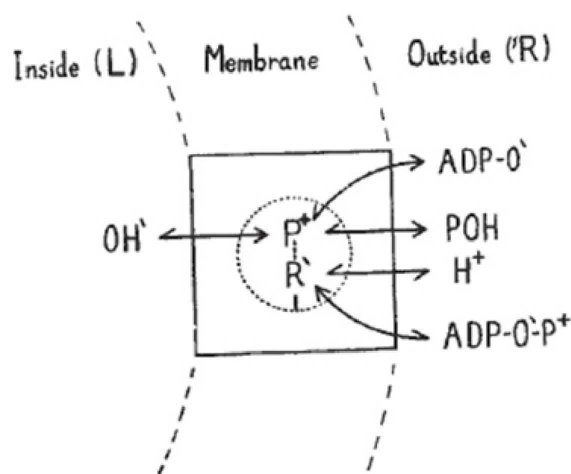

Figure 4. The original caption for this figure in Mitchell (1961) reads: "Fig. 1. Anisotropic reversible 'ATPase' system located in an ion-impermeable membrane between aqueous phases L and R." *Credit:* Reprinted by permission from Macmillan Publishers Ltd: *Nature* ©1961.

Mitchell knew that different metabolic inhibitors could inhibit oxidative phosphorylation, and he tentatively identified some of the components in his hypothetical system (Figure 5). He hypothesized that a flavoprotein, a quinone, and a cytochrome were important in oxidative phosphorylation. He placed these macromolecules within or adjacent to his membrane, based on the knowledge at the time. Please note that the notation for some molecules was different in 1961.

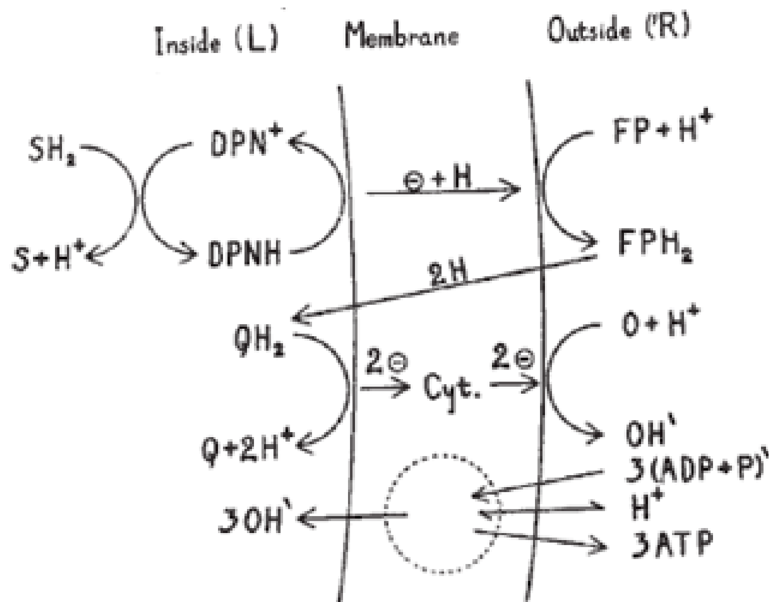

Figure 5. The original caption for this figure in Mitchell (1961) reads: "F.ig. 3. Diagram of chemi-osmotic system for coupling phosphorylation to the oxidation of substrate (SH_2) through DPN, *FP* (tentatively identified with flavoprotein), *Q* (tentatively identified with a quinone) and the cytochromes (*Cyt.*)." *Credit:* Reprinted by permission from Macmillan Publishers Ltd: *Nature* ©1961.

Questions

5. Use Figure 5 above to complete the following:
   a. Identify the following components: flavoprotein, quinone, and cytochrome.
   b. Circle the protons.
   c. Draw boxes around the electrons.
6. In Figure 5 does a proton or an electron cross the membrane first?
7. Is the cytochrome translocating protons or electrons?
8. In Figure 5, how many ATPs are made via the spatially positioned system?
9. Do you know how many ATPs scientists currently think are made with the electron transport chain?

Mitchell concluded his paper by stating:

> *"The underlying thesis of the hypothesis put forward here is that if the process that we call metabolism and transport represent events in a sequence, not only can metabolism be the cause of transport, but also transport can be the cause of metabolism."*

Mitchell understood that his proposal was contrary to the conventional biochemical approach. It took several other experiments to move the scientific community towards accepting Mitchell's hypothesis.

Part II – What Do They Do?

Paper #2: Jagendorf, A. and E. Uribe. 1966. ATP formation caused by acid-base transition of spinach chloroplasts. *Proc. Natl. Acad. Sci.* 55: 170–177.

While at Johns Hopkins University, Andre Tridon Jagendorf and Ernest Uribe generated ATP in an experimental system without cells and without metabolism just by changing the pH of solutions of membranes and allowing them to equilibrate (Figure 6). This series of experiments showed that differences in pH (and thus charges) across membranes could drive ATP synthesis.

They isolated chloroplasts from spinach and kept them in the dark so that no ATP would be formed from light. They put the isolated chloroplasts in a series of solutions at different acid pH's to start the experiment and then raised the pH of the solution.

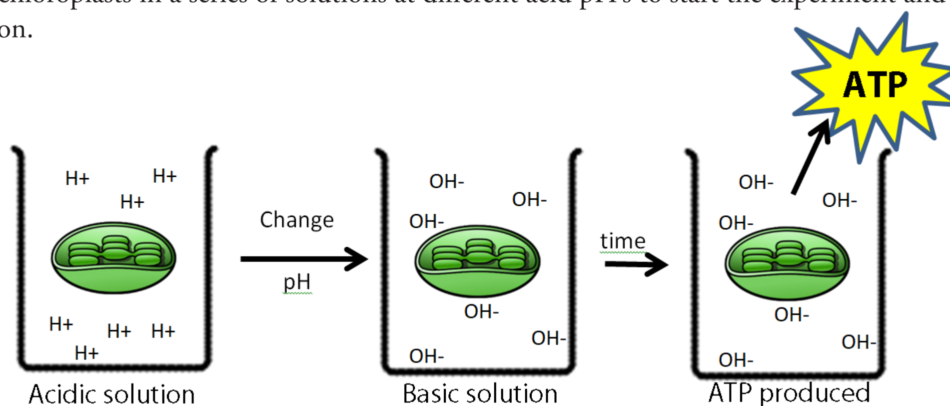

Figure 6. Jagendorf and Uribe's experimental setup.

Depending on the starting or ending pH, they found more or less ATP was formed. They postulated that when there is a charge gradient across the membrane (like the electrochemical gradient found when the outside and the inside were at different pH's), this was a high energy condition that could be used to drive ATP synthesis.

A figure from their paper (Figure 7) shows the amount of ATP that was produced by chloroplasts with different starting (acid stage) and ending pH's. Each datapoint is a different experiment with a different starting and ending pH.

Questions

1. What is measured on the X-axis?
2. What is measured on the Y-axis?
3. What was the starting and ending pH that gave the most ATP?
4. Does a large difference or a small difference in pH yield more ATP?

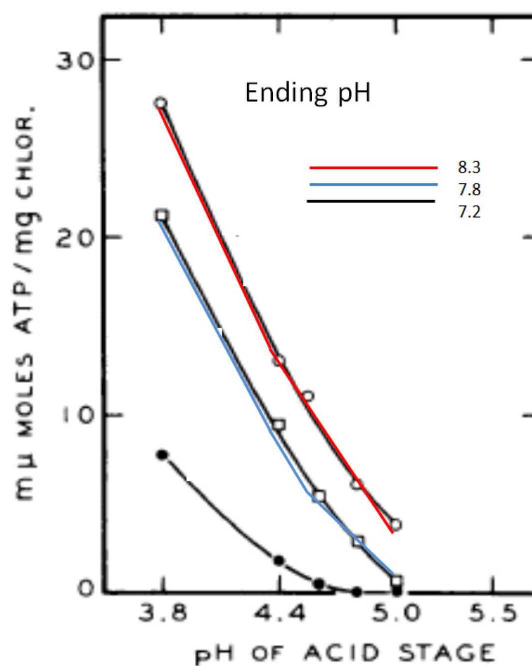

Figure 7. The original caption for this figure reads: "Fig. 3.—Yield of ATP as a function of acid stage pH." *Credit:* Adapted from Jagendorf and Uribe (1966).

Another graph from their paper (Figure 8) shows the amount of ATP produced by isolated chloroplasts when the starting pH was 4.0.

Questions

5. What is measured on the X-axis?
6. What is measured on the Y-axis?
7. Which pH yielded the most ATP?
8. Does there appear to be an optimum pH difference for ATP yield? What reason can you give for this?

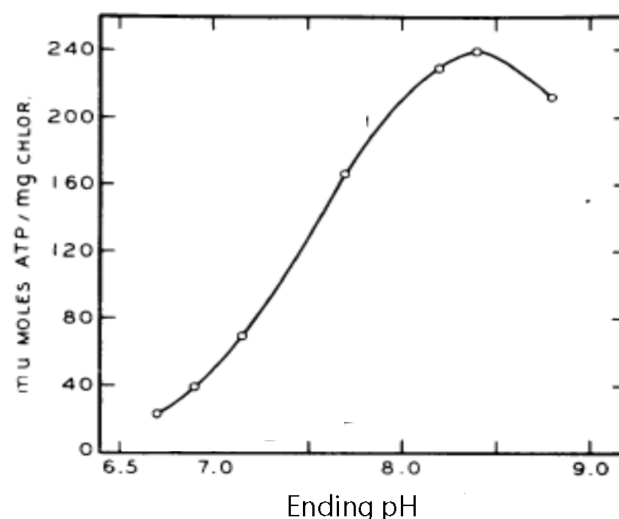

Figure 8. The original caption for this figure reads: "Phosphorylation stage pH curves, with differing amounts of succinate (pH 4.0 in each case) in the acid stage. Each amount and pH of succinate was neutralized by an appropriate amount of NaOH, and the pH of the phosphorylation mixture was checked at 0°C." *Credit:* Simplified from Jagendorf and Uribe (1966).

The conclusion of their paper is that changes in pH can drive the enzyme(s) that are responsible for the synthesis of ATP, in the absence of any other metabolism. It was later that other scientists determined that this enzyme was ATP synthase.

Part III – How Do They Do It?

Paper #3: Racker, E. and W. Stoeckenius. 1974. Reconstitution of purple membrane vesicles catalyzing light-driven proton uptake and adenosine triphosphate formation. *Journal of Biological Chemistry* 249: 662–663.

By 1974, 13 years after Mitchell first hypothesized ATP formation via chemiosmosis, scientists were starting to put together the pieces that were necessary to prove the theory. However, membranes are very complex, with many proteins in the lipid bilayer membrane or attached to either the inner or outer membrane surfaces.

While at Cornell University, Efraim Racker and Walther Stoeckenius purified membrane proteins from a purple photosynthetic bacterium, *Halobacterium halobium.* The purple membrane of this organism has only one protein, bacteriorhodopsin (called purple protein at the time), which responds to light by transporting protons. They made a preparation of soybean lipids to make artificial membrane vesicles. They then inserted the purple protein into a preparation of soybean lipids, making artificial membrane vesicles (Figure 9).

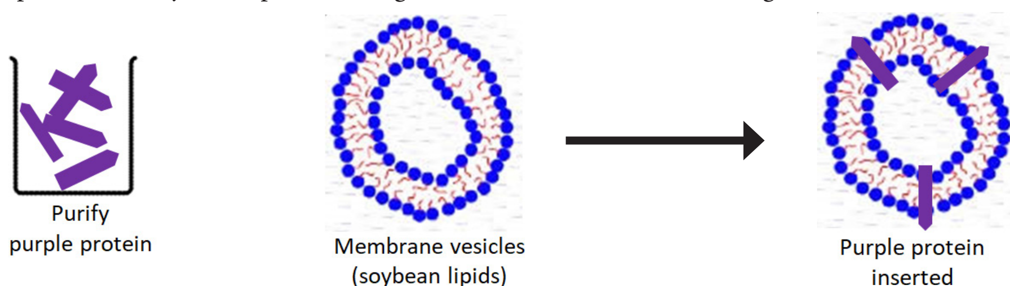

Figure 9. Racker and Stoeckenius' procedure for producing artificial membrance vesicles. Purified membrane proteins are obtained from the bacterium *H. halobium* and inserted into a preparation of soybean lipids.

When the membrane vesicles receive light (on), protons are transported. When the membrane vesicles do not receive light (off), protons are not transported. Proton concentration in the medium was measured by changes in pH (Figure 10).

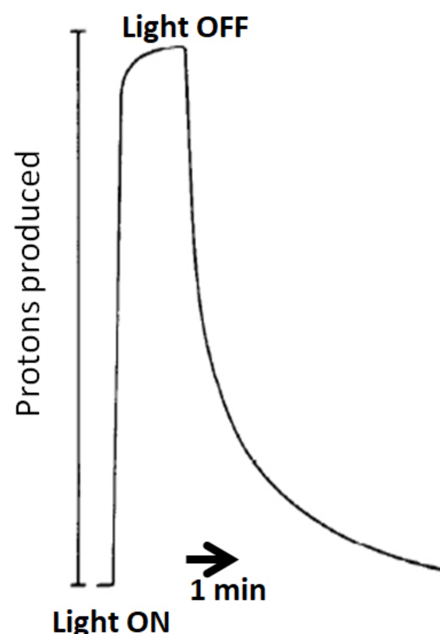

Figure 10. Response of membrane vesicles to light.

Questions

1. If the membrane vesicles were leaky would they have been able to measure changes in pH?

2. Does this experiment show that the scientists were successful in inserting the purple protein into the membrane? Why or why not?

Racker and Stoeckenius added different amounts of bacteriorhodopsin (purple protein) to the soybean phospholipid vesicles (Figure 11).

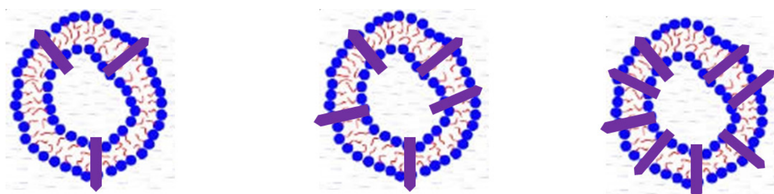

Figure 11. Increasing the amount of bacteriorhodopsin.

Their results are depicted in the graph to the right (Figure 12).

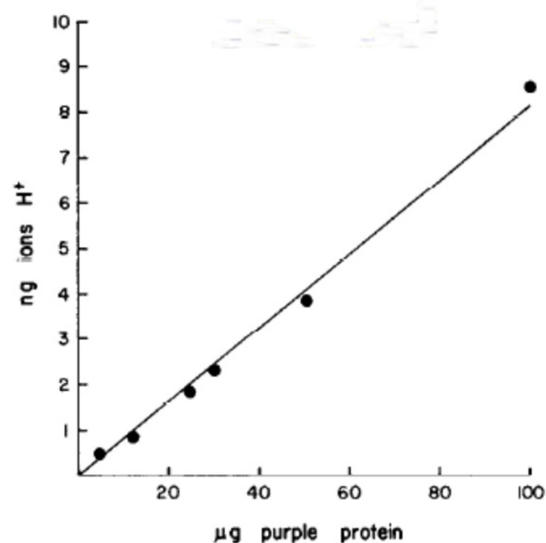

Figure 12. Results of adding different amouns of bacteriorhodopsin. *Credit:* Figure 2, Racker & Stoeckenius (1974).

Questions

3. What is the X-axis?

4. What is the Y-axis?

5. Does the amount of purple protein (or bacteriorhodopsin) affect the amount of protons that are transported?

6. Why did they do this experiment?

Racker and Stoeckenius took the next step and incorporated proteins from bovine heart mitochondria into their artificial membranes. We now know that the proteins were ATP synthase enzymes. They were able to detect ATP formation in the presence of light (Figure 13).

When they added oxidative phosphorylation inhibitors, no ATP was formed. No ATP was formed in artificial membranes without the bacteriorhodopsin.

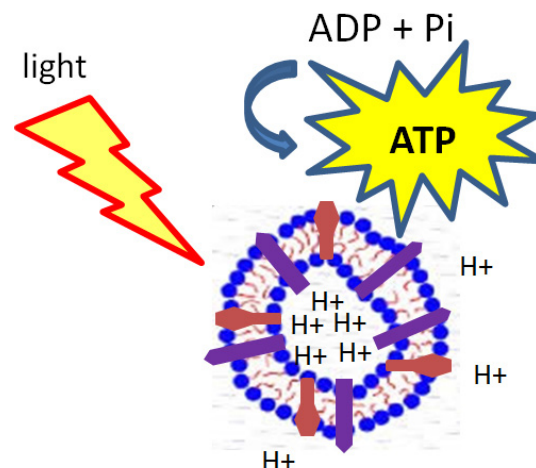

Figure 13. Protein from bovine heart mitochondria, symbolized in red, inserted into the artificial membranes.

Question

7. Why was it essential that both proteins were inserted in the membrane?

This model system provided evidence that the light-driven proton motive force generated by the bacteriorhodopsin drove the ATP synthase, generating cellular energy.

Part IV – Putting It Together

Compare and contrast Mitchell's 1961 model with the current model of the electron transport chain by reviewing Figures 14–16 and then answering the questions on the following pages.

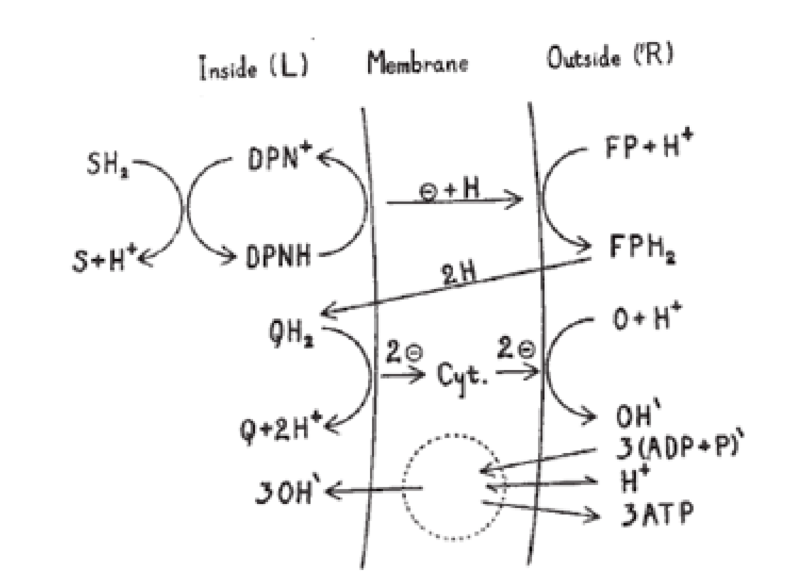

Figure 14 (same as Figure 4). Mitchell's 1961 model.
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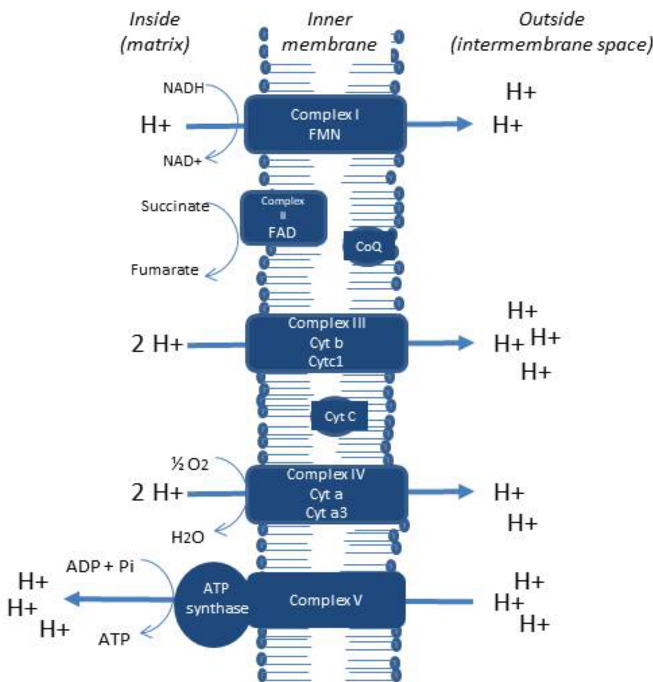

Figure 15. Current model for respiration.

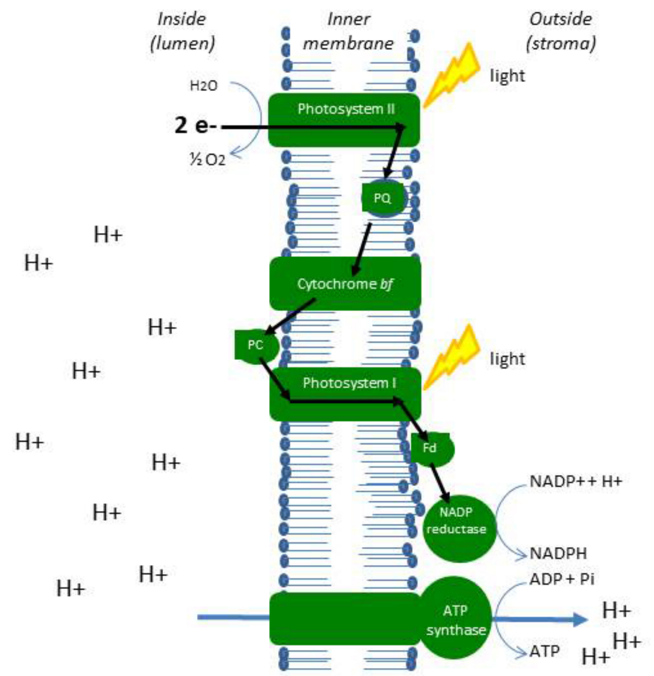

Figure 16. Current model for photosynthesis.

Questions

1. *Cytochromes:*
   a. How many cytochromes did Mitchell think there were?

   b. How many cytochromes are in the current model for respiration and what are their names?

2. *Flavoproteins:*
   a. What is Mitchell's abbreviation for flavoprotein?

   b. What is the current abbreviation for flavoprotein?

3. *Quinone:*
   a. On which membrane surface (outer or inner) does Mitchell have his quinone?

   b. Does the current model have the quinone on the inner or outer membrane surface?

   c. What is the name we call the quinone in respiration?

   d. What is the name we call the quinone in photosynthesis?

4. *ATP formation:*
   a. In Mitchell's model, what does the dotted circle at the bottom represent?

   b. Did he get the ratio of protons transported to ATP formed correct?

   c. Did he get the number of protons moved through the "mechanism" correct?

5. *Electron sources:*
   a. What are the different electron sources in photosynthesis and respiration?

   b. Was Mitchell's model for chemiosmosis based on photosynthesis or respiration? Why?

   c. What is the terminal electron acceptor for photosynthesis?

   d. What is the terminal electron acceptor for respiration?

6. *Spatial positioning:*
   a. What are the different names for the spaces outside and inside the membranes in chloroplasts and mitochondria?

   b. Where are protons pumped in chloroplasts versus mitochondria (inner membrane space versus outer membrane space)?

   c. Into which spaces is ATP synthesized in chloroplasts and mitochondria?