

Problem Set 5: Viruses

Due Tuesday, May 12 at 12:00 P.M. in the Bi 1 closet

<p>HOMEWORK INSTRUCTIONS</p> <ol style="list-style-type: none"> 1) Turn in your homework <u>stapled to this cover page</u>. 2) Use separate sheets of paper for your answers. 3) Write or type your answers neatly. 4) Put your name on each page of your answers. 5) Box your answers, please, so that the grader can find them. <p><i>Points may be deducted if you don't follow these instructions!</i></p>
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ANSWER KEY

Name: _____

Section #: _____

Mail Code: _____

TA Names: _____

Date and Time turned in: _____

Number of pages including this one: _____

AFTER YOU FINISH:

How long did it take you to complete this problem set? _____

Please also go to the Bi1 moodle site at <http://courses.caltech.edu> and take the homework survey.

There are **2** questions. The number of parts to each question is listed at the beginning of each; be sure to answer all the parts!

Grade:

Problem 1 _____

Problem 2 _____

TOTAL: _____

Problem 1: Tetherin (50 points – 9 parts)

The host-pathogen relationship is an old one. Considering this fact, it comes as no surprise that humans have acquired many defense mechanisms aimed at combating viruses. In turn, viruses have developed their own countermeasures aimed at bypassing host defenses.

In early 2008, Paul Bieniasz's group from Rockefeller University illustrated this point when his lab reported an interesting property of an HIV-1 mutant deficient in the virulence gene Vpu. Whereas infection with wild-type virus (which functions normally, and will be referred to as [HIV-1(WT)]) produced functional virions that were released after budding, a Vpu-deficient HIV-1 strain [HIV-1(delVpu)] had an abnormal budding phenotype in which virions were "tethered" to each other and to the surface of the host cell, rather than fully released. As a result, clumps of HIV-1(delVpu) virions accumulated at the infected cell surface. Figure 1 compares electron micrographs of HIV-1(WT) and HIV-1(delVpu) virions budding from infected cells.

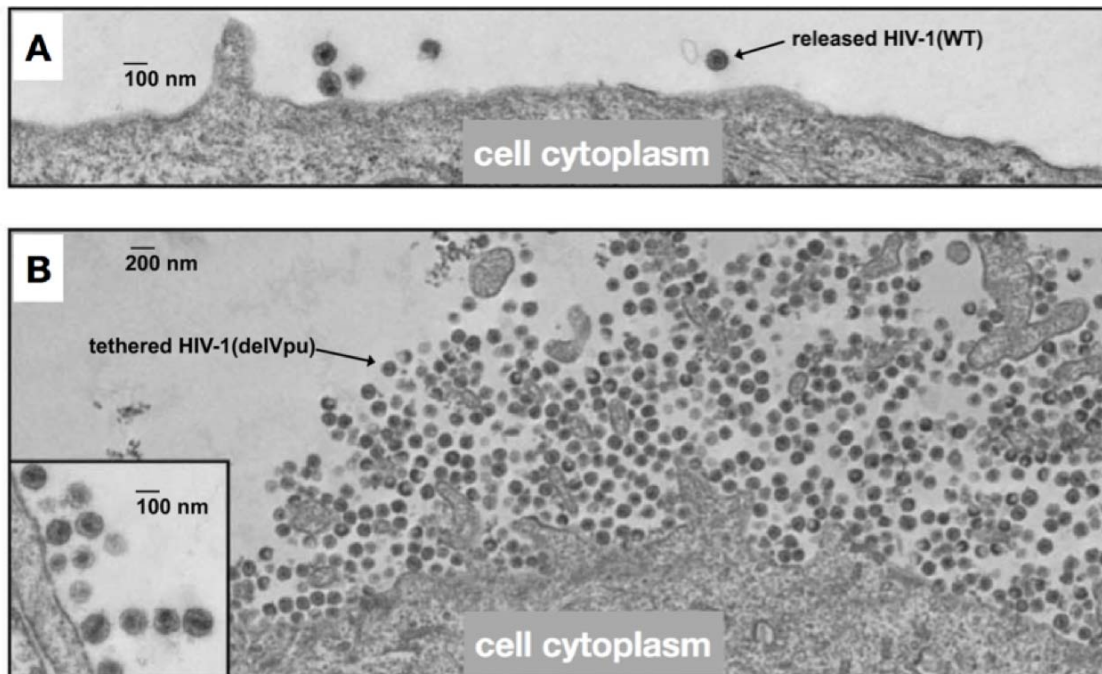


Figure 1. Arrows point to an example of a budding virion in each type of virus. (A) Section of a cell infected with HIV-1(WT). Viruses have budded and been released normally. Very few virions are seen because most have diffused away after release from the host cell membrane. (B) Section of a non-permissive cell infected with HIV-1(delVpu). Mutant viruses are tethered to each other and to the host plasma membrane and therefore accumulate in clumps at the cell surface.

Further investigation revealed that the abnormal budding of HIV-1(delVpu) occurred in only some cell types (cells that did not permit budding, or non-permissive cells); in other cell types (cells that permitted budding; permissive cells), HIV-1(delVpu) budded normally. However, HIV-1(WT) budded normally from both permissive and non-permissive cells. These results are shown schematically in Figure 2.

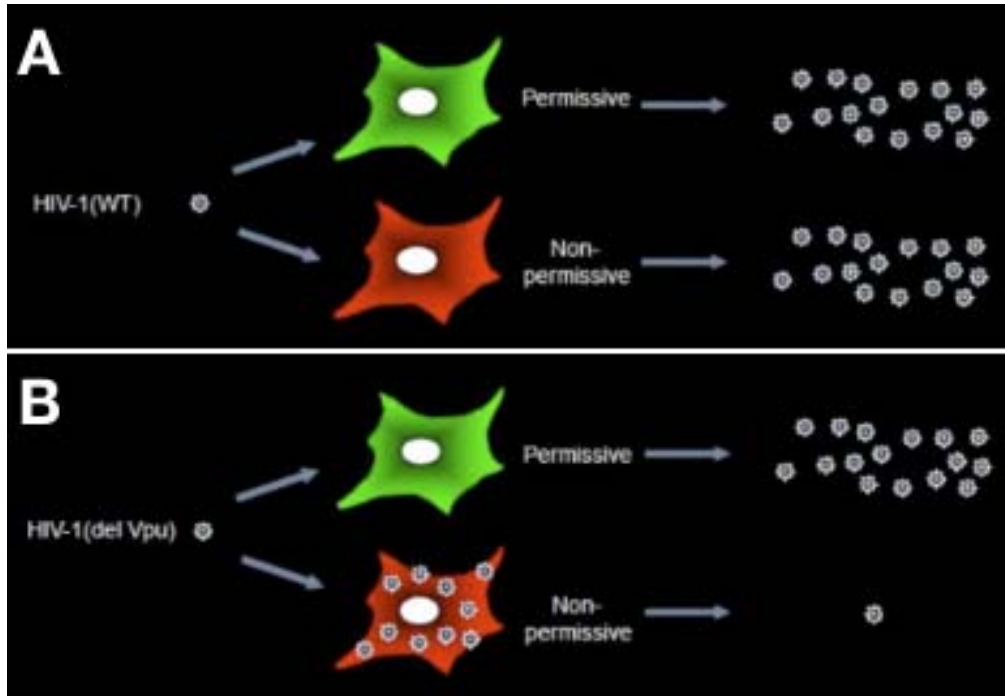


Figure 2. Schematic representation budding of **(A)** HIV-1(WT), **(B)** HIV-1(delVpu). The outcome of infection for both “permissive” and “non-permissive” cell types is shown for each condition.

A. (3 points) Using the data from Table 1 below, which cell lines can be considered permissive and which can be considered non-permissive?

HOS cells and HT1080 can be considered Permissive
 HeLa and 293T cells can be considered Non-Permissive

Cell line	Infectious virus release (% of WT virus in unmanipulated cells)	
	HIV-1(WT)	HIV-1(del Vpu)
HeLa	100	2
HOS	100	120
293T	100	7
HT1080	100	116

Table 1. Infectious virus release in different cell lines

Bieniasz’s group wanted to determine if the aggregation of virus particles at the surface of infected non-permissive cells was due to the presence of a proteinaceous factor at the surface of infected cells and viruses. Indeed, they found that treating HIV-1(delVpu)-infected non-permissive cells (e.g., as shown in Figure 1B) with a weak protease resulted in disintegration of viral aggregates and subsequent release of infectious virus. (The protease was added under conditions that could “shave” proteins off the cell and virion surfaces, but could not access cytoplasmic or other internal proteins.) This suggested that a surface protein causes the tethering

phenotype. They named this protein of unknown sequence and genetic origin **tetherin** after the ‘tethered’ phenotype it seemed to cause.

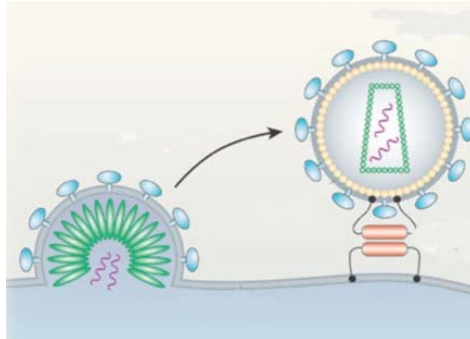


Figure 3. Proposed model for tetherin function in HIV-1(delVpu)-infected cells

B. (7 points) Interpret the schematic model in Figure 3 (i.e., write a figure legend explaining what is shown in the model). Address the following questions in your legend: By what mechanism could tetherin proteins appear on the virion surface as well as the host cell surface (assuming tetherin is encoded by the host cell)? Does Vpu facilitate tetherin function or does it inhibit tetherin function? Explain your reasoning. (5-6 sentences)

HIV buds from the host cell, acquiring viral RNA, capsid proteins and envelope proteins. Since HIV is an enveloped virus, it will acquire the host lipid membrane and associated proteins as it buds. HIV-1 (delVpu) viruses do not downregulate expression of host restriction factor tetherin. Tetherin is thought to homodimerize (have homophilic interactions) and ligate the virus to the host cell surface. As a result, viruses are tethered to the host surface and are unable to diffuse to other target cells. Tetherin thus abrogates infection by blocking viral spread.

Additionally, Bieniasz’s group found that treatment of cells with the cytokine Interferon-alpha (IFN- α) converted permissive cells into non-permissive cells. Cytokines are a group of small proteins that are released by cells and that have specific effects on cell-cell interactions, signaling, and/or cellular behavior. Interferon-alpha (IFN- α) is an anti-viral cytokine that is secreted by some immune cells when a viral infection (e.g., by HIV-1) is detected. Table 2, below, reports the results of an assay in which cells were grown in the absence of IFN- α (no IFN- α) or grown in the presence of IFN- α (+ IFN- α).

Cell line	Infectious virus release (% of WT virus in unmanipulated cells)	
	HIV-1(WT)	HIV-1(del Vpu)
293T (no IFN α)	100	7
293T + IFN α	100	6
HOS (no IFN α)	100	116
HOS + IFN α	100	1.4

Table 2. Effects of interferon- α treatment in different cell lines.

C. (5 points) What is the effect of IFN- α on viral release from permissive cell lines? What is the effect of IFN- α on viral release from non-permissive cell lines?

293T cells are non-permissive (like HeLa cells). IFN- α does not seem to affect non-permissive cells.

HOS cells are permissive (like HT1080) and IFN- α “converts” permissive cells into non-permissive cells.

D. (3 points) Which of the following hypotheses most likely explains the aforementioned results?

1. Permissive cells constitutively (always) express tetherin while non-permissive cells depend on IFN- α signaling to induce tetherin expression.
2. **Non-permissive cells constitutively (always) express tetherin while permissive cells depend on IFN- α signaling to induce tetherin expression.**
3. Both cell lines depend on IFN- α signals to induce tetherin expression.
4. Both cell lines constitutively (always) express tetherin regardless of IFN- α signaling.

To understand how tetherin functions, Bieniasz and colleagues set out to identify the gene encoding tetherin.

E. (6 points) Give one (or two for extra credit) reason(s) why identifying the tetherin gene might help in understanding how tetherin affects HIV-1 budding (i.e., What experiment(s) could you perform if you isolated the tetherin gene? How might knowing the protein sequence of tetherin help you decipher its function?) (3-4 sentences).

Once the gene causing the tethering phenotype is identified, we might find other functionalities attributed to this gene (Tetherin, or BST-2, is involved in B-cell development). The gene can also be recombinantly expressed in permissive cells to test changes in phenotype. Amino acid sequence can be determined from isolated and purified proteins. From this code, a nucleotide code can be deciphered and the putative gene sequences blasted against a database of known genes.

Note that at this point in time, the researchers had no methods to purify tetherin – all they knew was its expression pattern and that it was a surface protein. Since they couldn't use a protein sequence to help them find the tetherin gene, candidate tetherin genes were identified using a microarray analysis (see Lecture 9). mRNA was isolated from HeLa cells grown in the absence of IFN- α and from HOS cells grown in the presence and absence of IFN- α . Each mRNA sample was converted into a library of fluorescent cDNA targets, and the targets were used in dual color microarray experiments using a human genome gene chip as described in class. In one comparison, relative mRNA levels for several hundred gene transcripts were determined in HeLa cells and HOS cells grown in the absence of IFN- α . In a second comparison, the relative levels of mRNA for these gene transcripts were determined in HOS cells grown in the presence and absence of IFN- α . The results are shown in Figure 4 (on the next page).

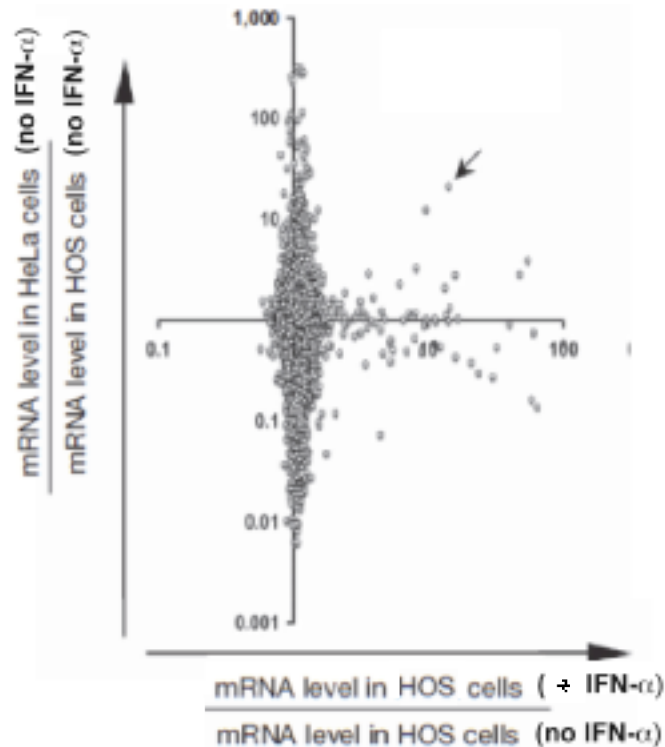


Figure 4. Data from two dual-color microarray experiments. Each dot represents the results for one transcript. The transcript most likely corresponding to the tetherin gene is indicated with an arrow.

F. (8 points) Explain why the data were plotted as “mRNA level in HeLa cells (no IFN- α)/mRNA level in HOS cells (no IFN- α)” (the ratio of the mRNA level in HeLa cells to the mRNA level in HOS cells when neither cell type was treated with no IFN- α) versus “mRNA level in HOS cells (+ IFN- α)/mRNA level in HOS cells (no IFN- α)”. You should answer the following questions in your response: Why would you expect the tetherin transcript to have a value greater than 1.0 on the vertical axis? Why would you expect the tetherin gene to have a value greater than 1.0 on the horizontal axis? Why is the transcript marked with the arrow most likely to represent the tetherin gene? (less than 10 sentences)

On the vertical axis HeLa cells are non-permissive and must express high levels of tetherin while HOS cells are permissive and should not express tetherin. Thus, the ratio of tetherin levels between these cell types will be greater than 1. On the horizontal axis, the ratio of tetherin levels are compared in permissive HOS cells induced to express tetherin by the addition of IFN- α versus permissive HOS cells without IFN- α and lacking tetherin. Thus the ratio of tetherin levels between these cell types will be greater than 1. Taken together, the gene to be identified will reside in the first quadrant of this plot, having a 1:1 correlation between the vertical and horizontal axes. The arrow points to the gene that fits these criteria best.

The researchers sequenced the gene corresponding to the transcript marked with an arrow in Figure 4. After translating the gene into its protein sequence, they performed a hydropathy analysis (similar to the analysis of bacteriorhodopsin that you performed in Problem Set 2). Figure 5 shows the hydropathy plot they obtained.

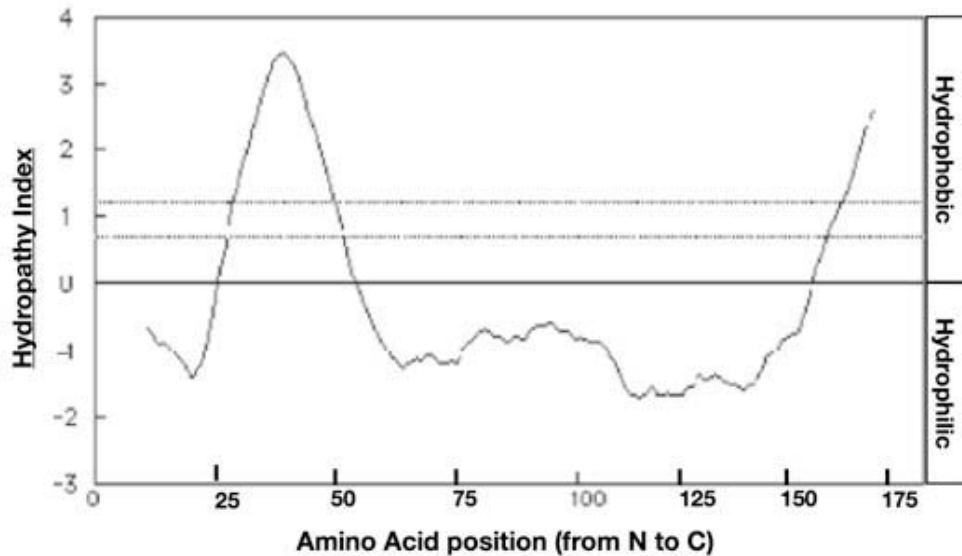


Figure 5. Hydropathy analysis of the tetherin sequence (you may refresh your memory about hydropathy plots by looking at Question 2 in Problem Set 2 again).

G. (5 points) From the hydropathy plot, which residues (list their numbers) most likely correspond to membrane-associated regions? Why?

The hydrophobic regions are between aa positions **25-50 (+/- 5)** and **150-175 (+/- 5)**.

The hydropathy analysis suggests that tetherin is a membrane protein with an unusual topology in which the protein is anchored to the membrane bilayer by two membrane-associated regions: one near the N-terminus and one at the C-terminus.

H. (6 points) Assuming that the bulk of the protein faces the extracellular space (rather than the cytoplasm), which amino acid positions correspond to the following regions of tetherin?

- i. (3 points) Cytoplasmic Domain** aa positions **1-25 (+/- 5)**
- ii. (3 points) Extracellular Domain** aa positions **50-150 (+/- 5)**

The predicted topology of tetherin suggested that its extracellular domain could engage in homophilic adhesion (i.e., self-self interactions) between membranes to explain its effects on budding of HIV-1(delVpu) virions.

I\$. (7 points) Studies of other viruses demonstrate that they have developed unrelated mechanisms for evading tetherin's effects on budding. For example, Ebola virus, which causes the severe hemorrhagic disease described in gory detail in The Hot Zone by Richard Preston, encodes a protein called GP that allows it to bud from tetherin-expressing host cells. Although GP functions similarly to Vpu, the protein sequences are unrelated. **Speculate on which evolved first, (i) the host tetherin function, or (ii) viral proteins that allow HIV-1 and Ebola to evade tetherin's effects. (3-5 sentences. Note there is no "correct" answer to this question. We will accept any answer that is logically reasoned. A speculation without accompanying an explanation will receive no credit**

A logically reasoned answer will obtain full-credit, so long as the hypothesis is supported by one or more pillars of evidence or logic.

It is likely that the host tetherin function evolved first, since we see several viral mechanical interpretations that each equally function to mitigate tetherin function.

Problem 2: Influenza virus (50 points – 8 parts)

Influenza (flu) is a serious respiratory disease. According to the CDC (Centers for Disease Control), 5-20% of the US population gets the flu each year. In this question, we will study influenza virus, the virus that causes flu, and the mechanism it uses to infect host cells.

This problem will require basic knowledge about the flu virus, which can be obtained from lectures and the book (chapter 35). The latter part of the problem concerns a classic experiment involving one of the flu virus proteins. You may find it useful to review the chemical properties of amino acids (chapter 3; p. 47-48), lipids/liposomes/detergents (chapter 6; parts 6.1, 6.2; figs 6.17, 6.21). It is also important that you understand endocytosis (chapter 7; p. 127-128; figure 7.15) because flu virus enters the cell via receptor-mediated endocytosis.

A. (2 points) Influenza A can be divided into different ‘subtypes’. Subtypes that are currently found in people include H1N1 and H3N2. The subtype behind the pandemic threat of bird flu is H5N1. **What are the differences between these three subtypes? To answer this, include the definitions of ‘H’ and ‘N’ and discuss why they are important to the virus.**

They have different combination of surface proteins. H= hemagglutinin and N= neuraminidase. Hemagglutinin is a surface glycoprotein that binds to host cell receptors and later is used in membrane fusion to release the viral content into the cytoplasm of the infected cell. Neuraminidase is used in viral exit. It cleaves sugar moieties on the cell surface to promote release of viral progeny from an infected cell.

B. (2 points) A new subtype of influenza A is a result of the process called ‘antigenic shift.’ There is also another process called ‘antigenic drift.’ **Explain these two terms (1-2 sentences each).**

Antigenic shift happens when the two different strains of viruses exchange their genomic RNA segments when they are in a secondary host. This results in new viruses with novel combination of H and N.

Antigenic drift is when mutations (usually point mutations) happen to alter the H gene. This results in changes in the viral epitopes such that existing neutralizing Abs cannot bind to H anymore.

Flu viruses enter host cells via receptor-mediated endocytosis. Later, the viral membrane fuses with the membrane of an endocytic vesicle. Subsequently, the virus releases its contents into the

cytoplasm. The viral protein that mediates membrane fusion is hemagglutinin (H). Now, we will take a closer look at hemagglutinin and the mechanism by which it promotes membrane fusion.

C. (10 points) In an early experiment to characterize the properties of flu hemagglutinin, scientists used a protease to release it from the viral membrane and then incubated it at various pHs before sedimenting it on a sucrose gradient (explained below). The cleaved form of H (referred to as the “ectodomain”) retained the portion of H responsible for fusion, but did not include the transmembrane-spanning region. By using only the H ectodomain for these experiments, the scientists could work with the protein in the absence of a detergent, which would normally be required to solubilize a membrane protein and prevent it from aggregating. Figure 1 (below), adapted from the PDB Molecule of the Month (April 2006) page, schematically shows hemagglutinin embedded in the viral membrane (the gray part).

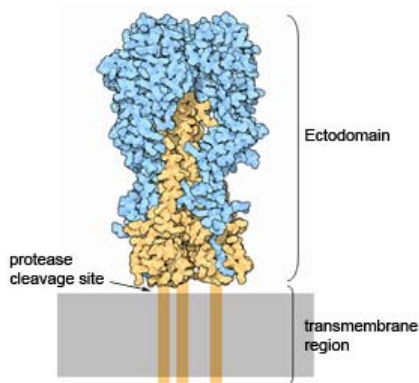


Figure 1. Hemagglutinin embedded in the viral membrane.

Sucrose gradient sedimentation separates molecules based on their molecular weights: large proteins with high molecular weights (e.g., aggregated proteins) migrate to the bottom of the gradient (fraction 1), whereas the native (unaggregated) form of H is found in fraction 12. In Figure 2 below (idealized from the real data), the absorbance at 280nm (A_{280}) for each of the fractions is plotted against the fraction number. Different symbols refer to sedimentations conducted at different pHs.

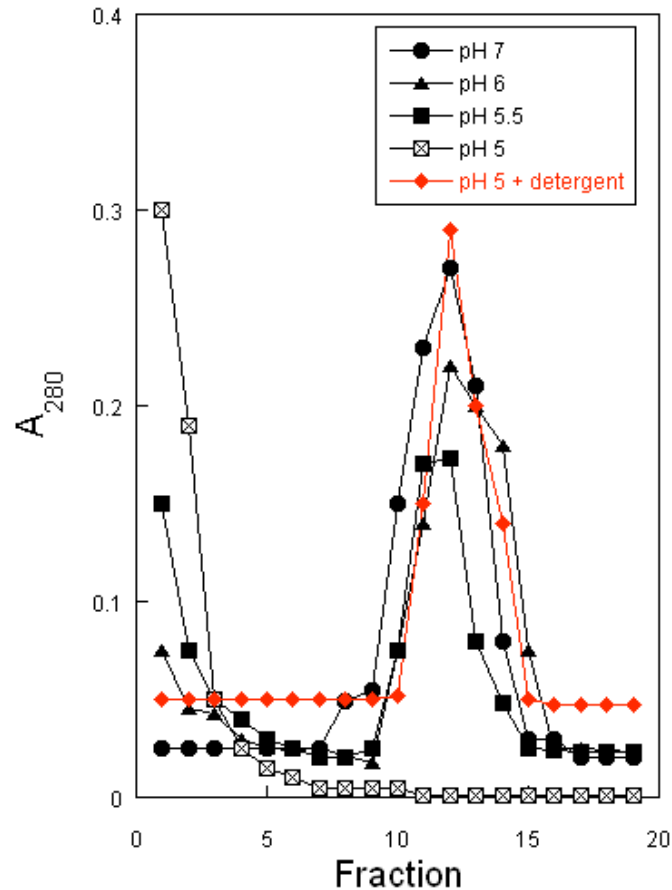


Figure 2 Sucrose gradient fractions

- i. (3 points) What kind of molecule is detected in the A_{280} measurement in this experiment? What type of chemical structure within this molecule gives rise to an absorbance at 280 nm? What type of instrument is used to measure A_{280} ? Hint: You may find Box 10.2 in your textbook and the following website helpful: <http://www.ruf.rice.edu/~bioslabs/methods/protein/abs280.html>
 - A_{280} detects proteins (in this case, H)
 - aromatic residues, such as phenylalanine, tyrosine, tryptophan
 - UV spectrophotometer
- ii. (2 points) Look at the results in the absence of detergent (black data points). What happens to H as it is incubated at progressively lower pH?

H changes from a native structure (fraction 12) to something bigger (i.e., aggregate in fraction 1) as the pH gets lower.
- iii. (2 points) Describe the difference in results at pH 5.0 in the presence (red diamonds) and absence (open black squares with x marks) of detergent.

In the absence of detergent at pH 5, H is aggregated. In the presence of detergent at pH 5, it is not aggregated.

- iv. (3 points) Propose an explanation for what happens to H at low pH that explains the results presented in the graph. Hint: consider a property of a protein that could cause it to aggregate. Also, consider the chemical properties of detergents and how detergents can function to prevent aggregation of membrane proteins.

At low pH, H changes its conformation to expose a hydrophobic segment (the fusion peptide) that causes H to aggregate unless the fusion peptide is inserted into a membrane or is complexed with detergent. Detergents solubilize membrane proteins (or in this case the low pH form of H; look at Fig 6.21 in your book.) by binding to exposed hydrophobic segments, thereby preventing aggregation. In the presence of detergent, H remains unaggregated, so a peak is not observed in fraction 1.

D. (8 points) The scientists further characterized the shape of H. Figure 3 shows two electron micrographs of H incubated at different pHs in the absence of detergent. These are “negative stain” images, in which protein appears where the stain does not, so proteins appear white. (The figures may look better on a computer than when printed out.)

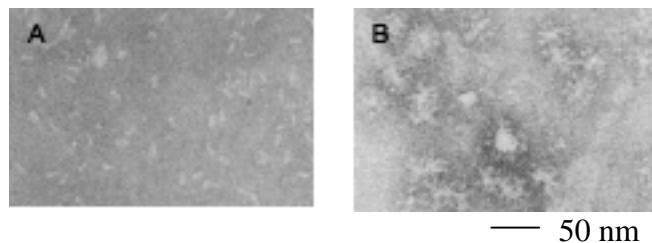


Figure 3 Electron micrographs of hemagglutinin

- i. (4 points) Based on the data from the sucrose sedimentation experiment above, which image corresponds to the pH 7.0 incubation and which corresponds to the pH 5.0 incubation? Explain.

A is pH 7 and B is pH 5.

From question 1, you can see that at low pH, H becomes aggregated and travels faster (because it is bigger) to the bottom of the gradient. In these micrographs, the bigger proteins are in B.

- ii. (4 points) How would incubation with detergent affect the micrographs in panels A and B? Describe expected changes in each, if any.

Panel A would not change. Panel B would look like panel A because aggregates would be disrupted by detergent.

E. (10 points) Next, the researchers investigated biochemical properties of H as a function of pH by studying its interactions with membrane bilayers. As a model membrane system, they used liposomes. A liposome is a spherical vesicle composed of a membrane bilayer formed by phospholipids. The membrane bilayer of a liposome is similar to the membrane of a cell or the

membrane of an intracellular organelle. The properties of liposomes are illustrated below in Figure 4 and in your textbook.

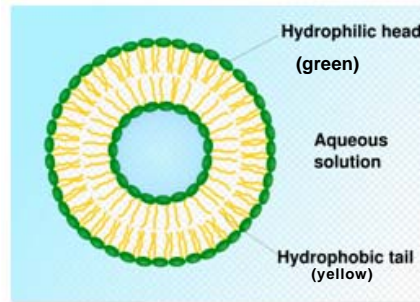


Figure 4 Schematic of a single liposome

For these experiments, the researchers incubated the H ectodomain with radiolabeled (radioactive) liposomes at different pHs and repeated the sedimentation experiment. They collected each fraction and assayed for the presence of lipid by measuring radioactivity (●). They also assayed for the presence of protein by using a dye that binds to proteins and can be detected by absorbance at 595 nm (A_{595}) (○). Study the results of this experiment (Figure 5) and answer the following questions:

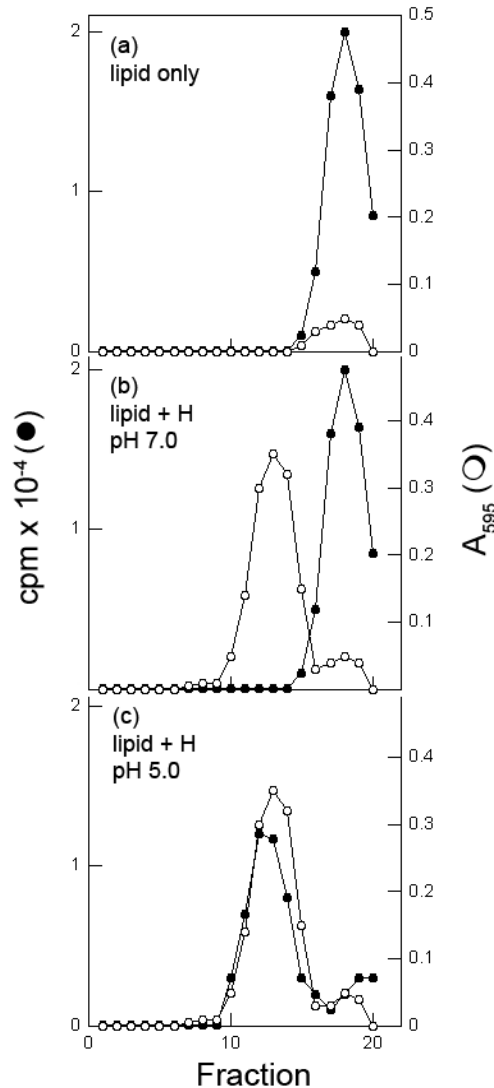


Figure 5 Sucrose gradient fractions

- i. **(2 points)** In the first sedimentation experiment (Figure 2), the researchers detected H by measuring the A_{280} of each fraction. **Why did they use a dye in the experiment shown in Figure 5 to shift the detection wavelength to 595 nm (the visible region)?**
Because lipid also absorbs at 280 nm.
- ii. **(2 points)** What is the purpose of the experiment in panel (a)?
Panel (a) is a negative control to see if the dye used to detect proteins binds to lipids. A small peak is observed at fractions 19-20 when the dye is incubated with lipids alone, which results from residual binding of the dye to lipids. The results of the experiment in panel (a) indicate that you should subtract the small peak observed in the absence of protein from the peak obtained in the panels from experiments conducted in the presence of protein.
- iii. **(3 points)** Explain the results in panels (b) and (c). Specifically, explain why the peak for H and the peak for the lipids coincide in panel (c), but not in panel (b).

At pH 7, the protein and the liposomes come out in different fractions → they don't bind to each other. However, at pH 5, the peaks coincide → H associates with the liposomes, so they are in the same fraction.

iv. (3 points) Explain why the results of this experiment are consistent with the results of the sedimentation experiment in question C. (Hint: consider the chemical properties of lipids and detergents.)

Both experiments suggest that H undergoes a conformational change at pH 5 that results in exposure of a hydrophobic segment that binds to detergent (first sedimentation experiment) or to lipids (second sedimentation experiment).

F. (6 points) Recall that flu virus first enters cells via receptor-mediated endocytosis. Describe a model based on the results in parts C, D, and E for how flu viruses deliver their genetic material to an infected cell. Include why it is advantageous for H to have different properties at acidic (pH ~5) versus neutral pH (pH ~7). Hint: consider what specialized cellular compartment to which the flu virus is delivered after it has entered the cell.

At low pH, H changes conformation to expose a hydrophobic region (fusion peptide) that causes it to aggregate (as seen in the EM) in the absence of detergent. This is consistent with the binding of H to detergent and to liposomes at low pH.

This is advantageous for the flu virus because H requires low pH to become fusion active, a flu virus will only fuse when it is in an acidic endosome (which means that the virus is inside a host cell). The virus will therefore be prevented from releasing its genetic contents prematurely.

G. (6 points) Proteoliposomes are liposomes that have membrane proteins incorporated into their lipid bilayers so that they can be used as simplified "cells" for certain types of experiments. Assume that you have the ability to make one set of proteoliposomes in which H is incorporated into the liposome bilayer, and another set of proteoliposomes in which the H receptor (a receptor for hemagglutinin) is incorporated into the liposome bilayer. When H binds its receptor under the right conditions (which you should know from answering the previous questions), it will trigger fusion of its membrane bilayer with the membrane bilayer of the receptor.

Assume that you can trap molecules inside each of the two types of proteoliposomes (e.g., you can trap molecule X inside the H proteoliposomes and molecule Y inside the H receptor proteoliposomes) so that the contents of the proteoliposomes will mix upon fusion (refer to schematic in Figure 6 below).

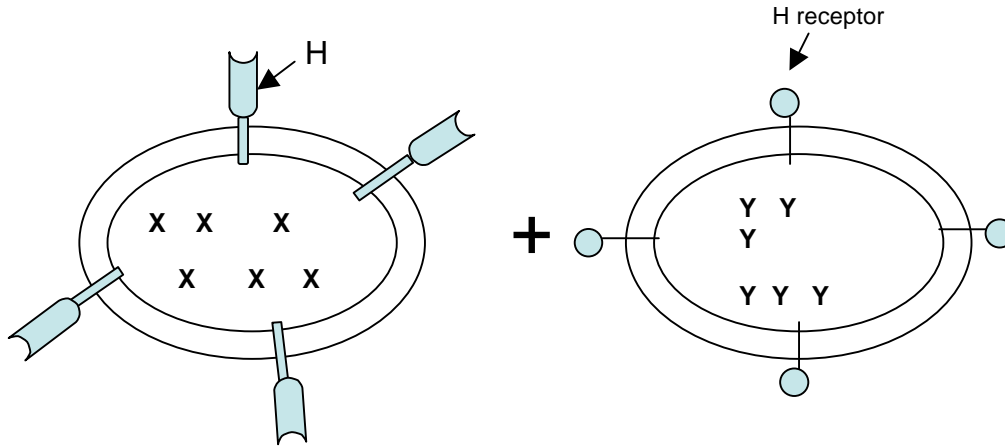


Figure 6 Schematic of proteoliposomes with incorporated H and H receptor proteins

Describe an assay whereby you can detect fusion of the H proteoliposomes with the H receptor proteoliposomes - i.e., tell us an example of what you could use for molecules X and Y. Be sure to discuss what conditions you would expect to see fusion, your expected results, and what controls (both positive and negative) you would have to include to make sure your assay is really monitoring fusion.

Classic example is put RNA in one liposome and RNase in the other, then look for degradation of RNA at low pH. Could also use protease and protein, fluorescent donor and acceptor, fluorescent probe and quencher, etc.

Positive control is the amount of degradation or fluorescence when you add detergent to the proteoliposomes or just mix X and Y together.

Negative control is amount of degradation or fluorescence at neutral pH.

H. (6 points) Would you expect HIV envelope spike proteins to have different properties at acidic (pH ~5) and neutral pH (pH ~7)? Why or why not? (<5 sentences)

No, because HIV fuses at the plasma membrane where the pH is constant.

Please remember to enter the number of hours you spent on the set on the cover page and submit your comments to the Bi1 moodle site.

<http://courses.caltech.edu>