History of the Analysis of Hemoglobin and Sickle Cell Anemia

During this activity, you will be exploring the scientific foundations of protein research and both the genetic and biochemical basis for the disease Sickle Cell Anemia. Analyze the timeline below. Read and discuss the papers and data provided by your instructor. Read the prompts and follow the directions provided on this sheet in order to analyze the data and answer the questions that follow.

An historical perspective:

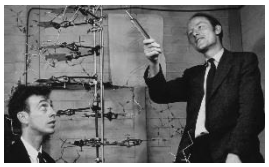
Gregor Mendel first published his research on inheritance in 1865. Fast forward 88 years to 1953 when Watson & Crick published their famous paper reporting the structure of DNA. In the years surrounding Watson & Crick's research, much was going on in the world of genetics and protein discovery.

***Vernon Ingram**, among others, did a great deal of research on hemoglobin and the gene mutation that causes Sickle Cell Anemia. Keep in mind, however, that the central dogma of biology (DNA → RNA → Protein) and the exact relationship between DNA and protein, while discussed and hypothesized, had not yet been solidified at the time of this research.*

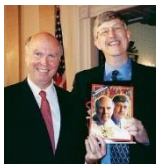
1865: Gregor Mendel publishes research on inheritance.

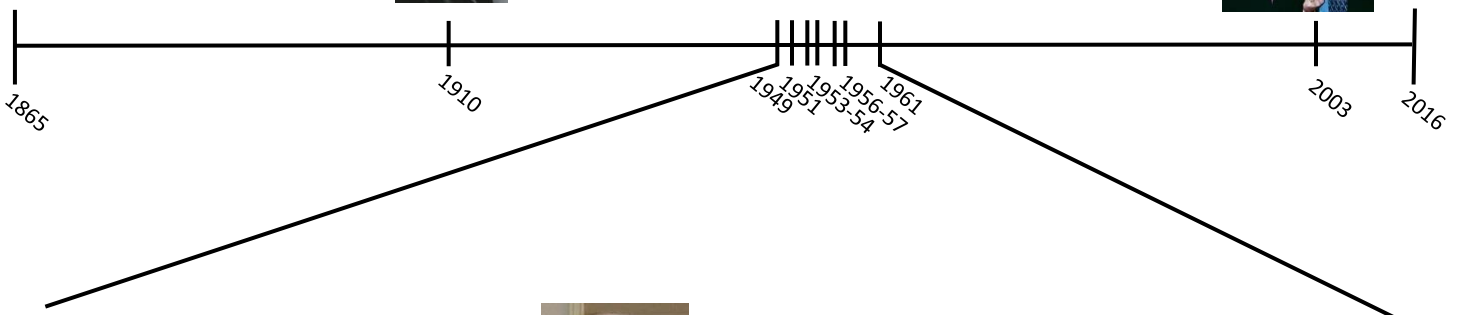
1910: James Herrick is first to document sickle cell anemia.

1953: James Watson and Francis Crick publish paper on structure of DNA.



2003: J. Craig Venter and Francis Collins complete Human Genome Project.





1949: Jim Neel showed sickle cell anemia is caused by homozygosity for a gene; Linus Pauling called sickle cell anemia the first "molecular disease."

1954: Tony Allison described the heterozygote advantage of sickle cell trait.

1961: Corrado Baglioni improves tryptic digest of hemoglobin; confirms difference in peptides.

1951: Fred Sanger & Hans Tuppy demonstrated for the first time that a protein, insulin, was composed of a specific sequence of amino acids.

1956-57: Vernon Ingram compares the tryptic digests of normal and sickle cell hemoglobin.

Timeline:

- ***1865-*** Gregor Mendel published his research on inheritance
- ***1910-*** James Herrick is the first to document sickle cell anemia
- ***1949-*** 1) Jim Neel demonstrated that sickle cell anemia is caused by homozygosity for the sickle cell gene. Today, we know that gene is actually the ***beta-globin*** gene. 2) Linus Pauling called sickle cell anemia a "molecular disease," and showed that the Hb-S (sickle cell beta-globin) protein had a 2+ to 3+ charge difference compared to the Hb-A (normal beta-globin) protein
- ***1951-*** Fred Sanger & Hans Tuppy solved the first complete amino acid sequence of a protein; the B chain of Insulin
- ***1953-*** James Watson & Francis Crick published their famous paper describing the structure of DNA
- ***1954-*** Tony Allison described the relationship between malaria and sickle cell anemia and determined there to be a "heterozygote advantage" for carriers of the sickle cell trait in malaria-stricken areas
- ***1956-1958-*** Vernon Ingram performs tryptic digests and publishes several papers comparing the structure of normal human hemoglobin to mutant sickle cell hemoglobin

Recall that during this time (mid 1950's), the exact relationship between genes and proteins had not yet been fully elucidated, and methods of protein and amino acid analysis were very limited compared to today's methods like X-ray crystallography and solution NMR. Vernon Ingram was at the forefront of developing and improving protein analysis techniques.

You have previously learned that the hemoglobin protein is actually made of four protein chains; two alpha chains (α-globin) and two beta chains (β-globin). However, during the time of Ingram's research, it was not known that hemoglobin consisted of both α-globin and β-globin chains. Rather, it was only known that hemoglobin was made up of "two identical half-molecules." Later, we would learn that each half-molecule has an alpha and a beta chain, and only the β-globin chain carries the mutation for sickle cell anemia. Individuals afflicted with sickle cell anemia are homozygous for this mutation. Those who are heterozygous for the mutation (carriers) are said to have sickle cell "trait," rather than the full anemia.

A. ***Picture it: Cambridge, England, 1954.*** You are a graduate student working with Vernon Ingram in Max Perutz's lab at the Cavendish Laboratory. Right next door is James Watson and Francis Crick; famous for their description of the structure of DNA. You've just learned of Linus Pauling's work showing the charge difference between the normal hemoglobin protein, Hb-A, and the sickle-cell protein, Hb-S. However, little is still known about the structural change in hemoglobin that leads to sickle cell anemia. Ingram charges you with the task of designing an experiment to better analyze hemoglobin structure, and to establish the molecular basis of sickle cell anemia.

What ideas can you generate about a potential method for analyzing and comparing the sequence and structure of proteins? Keep in mind, the molecules within proteins are generally too small for even the most powerful microscopes to view. Brainstorm some ideas. Discuss these ideas with your classmates.

Record your ideas here:

B. ***Understanding Protein "Fingerprinting"*** Prior to answering the following questions, read the following papers by Vernon Ingram:

- *Abnormal Human Haemoglobins, I. The comparison of normal human and sickle-cell haemoglobins by "fingerprinting." Biochimica Et Biophysica Acta,* Vol. 28, pg. 539-545 (1958)

Conduct some of your own research on the enzyme trypsin, and how Ingram's 'tryptic digest' technique broke down the protein in order to further analyze it? Where, exactly, does the enzyme trypsin digest (break or cut) the peptide bonds in a protein? (For additional information on tryptic digest technique, see the Resources page)

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Based on your research, you know that trypsin is a protein that breaks down proteins. What types of potential issues might this create? How might the protein or the organism have evolved to cope with or counteract this potential issue? Brainstorm ideas here. Do additional research on trypsin to see if you can determine this.

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C. *Analyzing a tryptic digest* Examine Figure 1 below before answering the following questions.

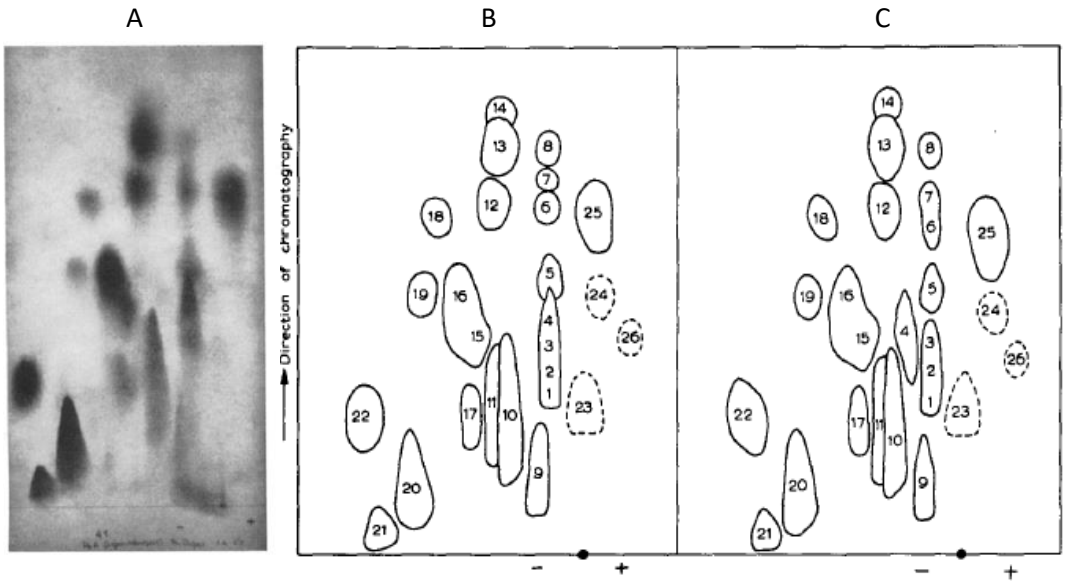

Figure 1. Tryptic digest data from Vernon Ingram in 1958. Peptides of hemoglobin A and S were subjected to both electrophoresis and chromatography in order to separate and classify the chemical identity of each peptides. Electrophoresis data is displayed on the horizontal axis, which indicates the relative charge of the peptides. The – and + symbols represent the charges applied to the electrophoresis paper. Chromatography data is displayed on the vertical axis, indicating the solubility of each peptide. The two combined represent a 2D "fingerprint" of the protein. Shown are the peptide fingerprint of normal hemoglobin **(A)** and tracings of normal **(B)** and sickle cell hemoglobin **(C)** fingerprints. Black dots indicate starting position. Numbers are randomly assigned for normal hemoglobin and then paired for sickle cell hemoglobin. (*Figure 3 from Ingram, 1958*)

Analyze Figure 1 above and answer the following questions.

- In the past, you may have learned about the process of electrophoresis and its application in biotechnology. Based on your knowledge of electrophoresis, analyze the location of the peptides in the medium. What can you assume are the net charges of peptides #22 and #25, respectively? Explain.

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- You also may have previously learned about the principles of chromatography. Based on that prior knowledge, and the location of the peptides in the medium, which is likely more soluble in the chromatography solvent; peptide #8 or #9? Explain.

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- What similarities and/or differences do you see in Figure 1, the 2D fingerprints of normal hemoglobin vs. the fingerprint of mutant sickle cell hemoglobin?

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- Brainstorm and discuss the potential causes for these changes in the hemoglobin protein observed by Ingram. Can you think of multiple different scenarios? Describe the potential causes here.

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D. ***Modeling a tryptic digest*** Observe the two amino acid sequences provided by your instructor. One is the amino acid sequence for normal beta-globin, while the other is the sequence for the mutant beta-globin that causes sickle cell anemia. Cut out each sequence, and tape them together in order to make a single linear sequence for each protein.

Determine where the trypsin enzyme would cut the proteins, and highlight those locations. Then model a tryptic digest by cutting them at those highlighted areas. Have your instructor check your work.

Based on your analysis of the sequences and tryptic digest, answer the following questions.

- In how many locations could trypsin cut each beta-globin protein? How did you determine this?

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- How many peptide pieces could the tryptic digest yield in each protein?

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- Compare the number of peptides you counted here, to the numbers reported in Figure 3 of Ingram's paper (or Part C, Figure 1 in this handout). Is there a difference? If so, account for this difference.

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E. ***The Amino Acid Substitution*** Analyze both the normal beta-globin amino acid sequence as well as the mutant (sickle cell) beta-globin amino acid sequence. Locate and highlight the amino acid substitution that occurred in the mutant protein as a result of the DNA mutation. Identify the substitution here.

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- Describe the biochemical differences between these two amino acids. How might that substitution impact the mutant beta globin protein structure?

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- Examine the genetic code for each amino acid involved in the substitution. What is the potential RNA genetic code substitution that occurred to cause the change in amino acids?

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Going further:
For advanced students or those seeking a greater challenge, consider the next two activities.

1. A Biochemistry Challenge:

Refer to part D above, *Modeling a tryptic digest*. Analyze the amino acid contents of each peptide. Within each peptide, determine what charges are present based on the chemical properties of the amino acids in each peptide. Mark any positively or negatively charged amino acids.

Next, perhaps re-read the methods section of the 1958 paper. Consider the solutions that Ingram used for both the electrophoresis and the chromatography. How might the pH of those solutions play a role in how the peptides distribute throughout the electrophoresis process? See if you can determine the overall net charge of each peptide, taking into consideration the pH of the solution they are in. It may be useful for you to determine the dissociation constant (pKa) of each of the charged amino acids. With this information, determine which peptides are the positively charged, and which are negatively charged. Predict their behavior in the electrophoresis process.

2. A Modeling Opportunity:

Using what you've learned about tryptic digest and mutant beta-globin, design a 3D printed model of mutant sickle cell beta-globin that distinguishes each peptide liberated during the tryptic digest, as well as the amino acid substitution that causes sickle cell anemia. In addition, you may also find it useful to display all the charged amino acids within each peptide.

REFERENCES

Baglioni, C., 1961. An improved method for the fingerprinting of human hemoglobin. *Biochimica Et Biophysica Acta* 48: 392-396.

Ingram, V., 1956. A specific chemical difference between the globins of normal human and sickle-cell anaemia haemoglobin. *Biochimica Et Biophysica Acta* 178: 792-794.

Ingram, V., 1958. Abnormal human haemoglobins, I. The comparison of normal human and sickle-cell haemoglobins by "fingerprinting." *Biochimica Et Biophysica Acta* 28: 539-545.

Ingram, V., 2004. Sickle-Cell Anemia Hemoglobin: The Molecular Biology of the First "Molecular Disease" – The Crucial Importance of Serendipity, *Genetics Society of America* 267: 1-7.

Stretton, A. O. W., 2002. The First Sequence: Fred Sanger and Insulin, *Genetics Society of America* 162: 527-532.

RESOURCES

- For more information on tryptic digest, read Ingram, V., 1956. A specific chemical difference between the globins of normal human and sickle-cell anaemia haemoglobin. *Biochimica Et Biophysica Acta* 178: 792-794., or try http://www.mcponline.org/content/3/6/608.full
- For more information on heterozygote advantage and the relationship between malaria and sickle-cell trait, try https://www.youtube.com/watch?v=ZsbhvI2nVNE
- To learn more about how the change in Hb biochemistry give rise to the morphological changes in the RBC, and eventually leads to sickle-cell disease, try https://www.youtube.com/watch?v=R4-c3hUhhyc