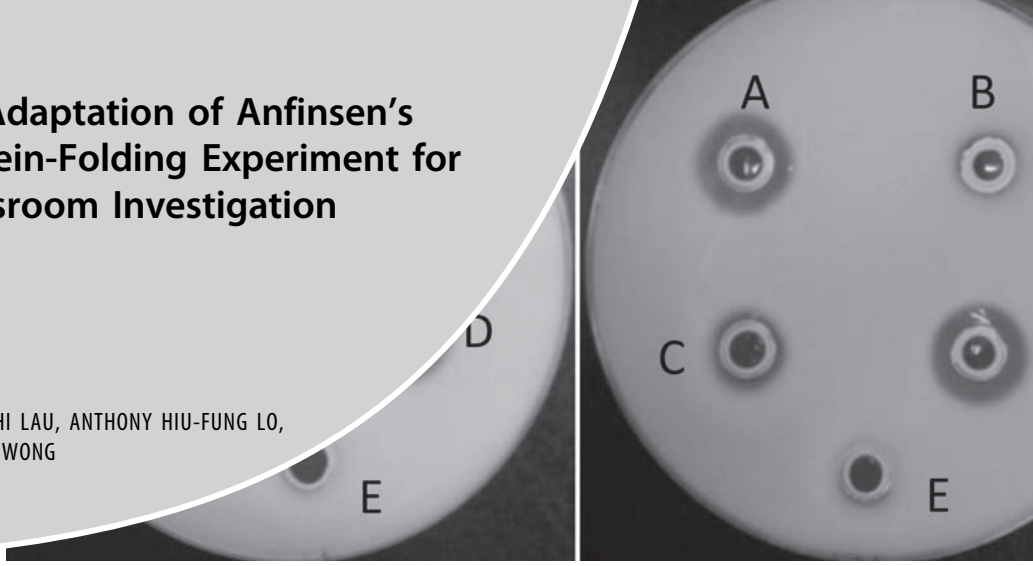
INQUIRY & INVESTIGATION

An Adaptation of Anfinsen's Protein-Folding Experiment for Classroom Investigation



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Abstract

The study adapts Anfinsen's Nobel-winning experiment of protein folding into biology investigation for secondary and college students. This experiment is significant for secondary and college science learning not only for its associations with some core concepts in biology, but also for its rich nature of science and science practices. The lab procedure of the original experiment was modified to be accessible to secondary biology teachers and students. A detailed lab manual and task sheet are available.

Key Words: *biology investigation; protein folding; Anfinsen.*

○ Introduction

The study will try to adapt Anfinsen's Nobel-winning experiment about protein folding (Anfinsen & Haber, 1961) into biology investigation for secondary and college students. This experiment is significant for biology learning not only for its associations with some core concepts in biology—genes, proteins, and traits (LS1 and LS3 in NGSS, 2013)—but also for its rich nature of science and science practices. The experiment, however, is not commonly done in secondary schools because the expertise and equipment required are beyond secondary levels, and no appropriate resources are available. This study will try to modify the experiment to make it accessible to most biology teachers and students.

Christian Boehmer Anfinsen received the Nobel Prize for Chemistry in 1972 for his investigation into how a protein folds into its native shape. He and his collaborators discovered that ribonuclease (RNase) could spontaneously refold back into its active shape after being fully unfolded by denaturants (Figure 1). This shows that all the information required for a polypeptide chain to fold into its active shape must reside in the amino acid sequence rather than be imparted from anything else during protein synthesis, such as DNA, RNA, ribosome, and enzymes. The correct refolding, however, only took place in an environment with specific pH and temperature. When urea is present to disrupt the hydrophobic environment, the unfolded proteins will only refold randomly into "scrambled" proteins that have no catalytic activity (Figure 1). Anfinsen (1973) further proposed a *thermodynamic hypothesis* to account for the protein folding: "the three-dimensional structure of a native protein in its normal physiological milieu (solvent, pH, ionic strength, presence of other components such as metal ions or prosthetic groups, temperature, etc.) is the one in which the Gibbs free energy of the whole system is lowest; that is, that the native conformation is determined by the totality of interatomic interactions and hence by the amino acid sequence, in a given environment" (p. 56). Anfinsen and others' work had established an important principle in biology that a gene determines the amino acid sequence, and the sequence in turn determines the active shape, and this shape ultimately determines the biological functions (Kresge et al., 2006) (Figure 2).

Misconceptions

The protein folding investigation can help tackle some common misconceptions associated with enzymes, proteins, and inheritance. In a study, 61 percent of secondary students considered genes as traits, 21 percent considereed genes as proteins and amino acids, while only 16 percent correctly stated genes are sequences of nucleotides (Marbach-Ad, 2001; AAAS project 2061, n.d.). Only 25 percent of students correctly answered that the information for making proteins comes from DNA, while 50 percent thought it is from amino acid and 25 percent from enzymes (AAAS project 2061, n.d.). There are 50% of students thought that DNA is made of proteins or amino acid (Marbach-Ad, 2001). This protein folding investigation can help clarify some of these misconceptions by engaging students in thinking about how information flows from DNA to proteins to traits.

The following concepts are the core ideas in the Next Generation Science Standards (NGSS, 2013):

- Genes are regions in the DNA that contain the instructions that code for the formation of proteins, which carry out most of the work of cells (LS1, grade 12).

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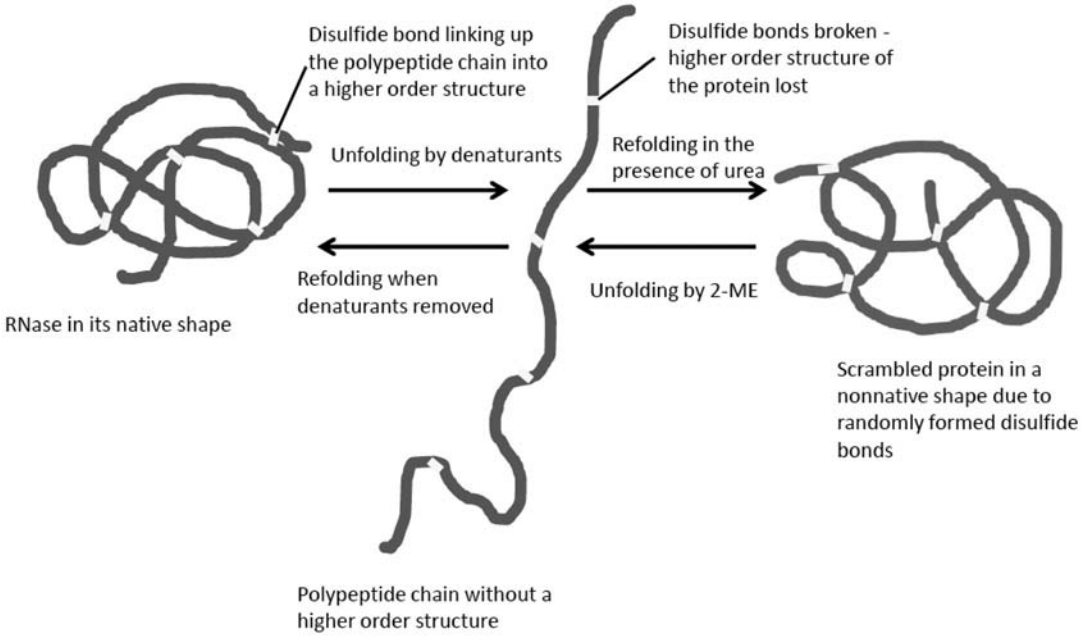

Figure 1. Outline of the RNase refolding experiments done by Anfinsen. RNase is first unfolded and then refolds back into its native shape when denaturants are removed. But in the presence of urea, RNase refolds into "scrambled" protein by randomly formed disulfide bonds.

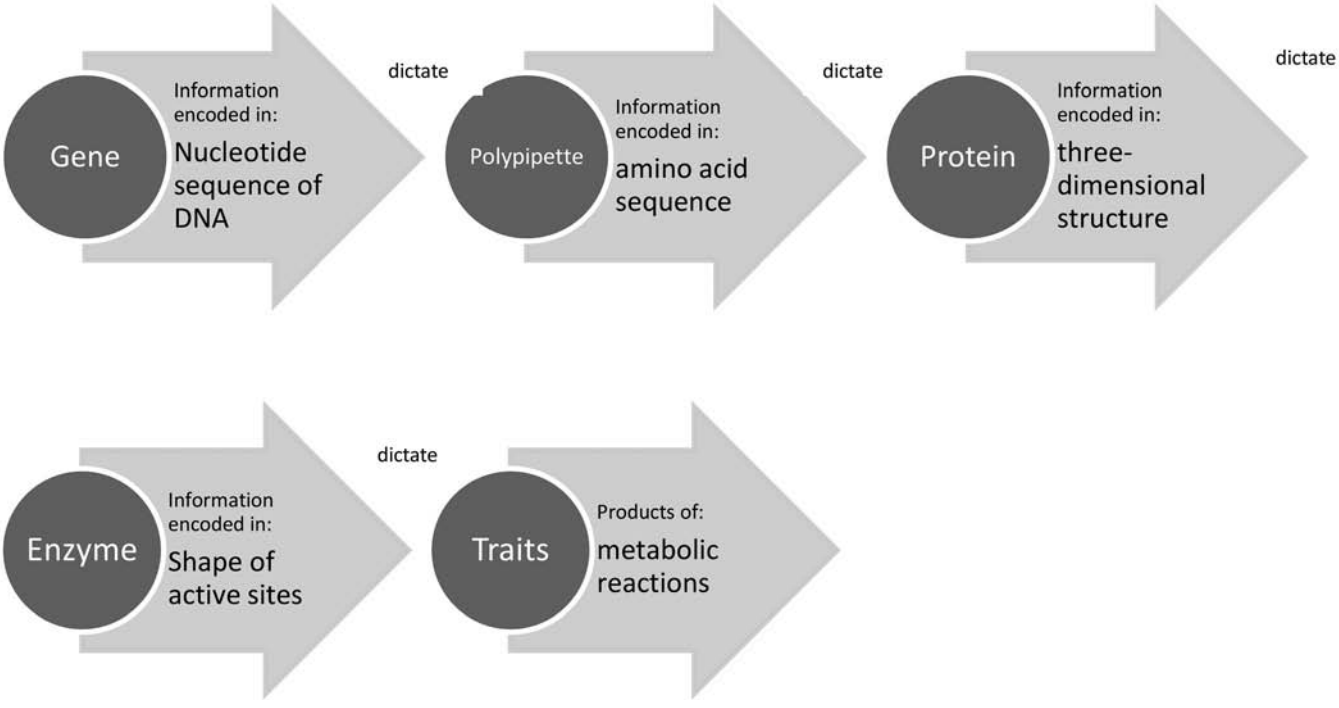

Figure 2. Flow and translation of information from genes to traits.

- Genes encode the information for making specific proteins, which are responsible for the specific traits of an individual (LS3).

These concepts are also highly relevant to AP biology on protein structure and functions (College Board, 2015, 4.A.1). The protein folding experiment adds an important concept to this chain of reasoning:

- Genes do not directly dictate the protein structure, but indirectly through determining its amino acid sequence that in turn dictates the higher order structure.

These concepts also give a good example on how biological structure is related to function, a crosscutting concept of NGSS.

Nature of Science

This investigation can be used to teach about many aspects of the nature of science. Enzyme refolding into its native shape is not directly observed but inferred from the observation that the enzyme regains its activity after denaturation. There could be alternative hypotheses that account for this observation: 2-ME and urea may act as enzyme inhibitors to alter the shape of the active site rather than unfold the protein completely; or, the RNase may not have refolded into its native shape but another shape that happens to have the same catalytic function as the native one. This poses the problem of *underdetermination* between scientific theory and evidence (Duhem, 1954). Nonetheless, these alternative hypotheses had been ruled out by further empirical evidence obtained from X-ray diffraction, UV spectrophotometry, chromatography, and electrophoresis that the structures and shapes of RNase do change during unfolding and refolding (White, 1961). Therefore, despite that scientific theory is inference and thus inherently tentative, it is nonetheless reliable in light of its multiple lines of empirical evidence.

The original question Anfinsen and his colleagues wanted to answer was not protein folding, but the catalytic activity of RNase. They cleaved the disulfide bonds of RNase with the aim of finding out if the bonds were related to its catalytic function. But accidentally, they found that RNase unfolded and refolded, opening up the research area of protein folding (Sela et al., 1957; White, 1961). This shows that scientific inquiry may not be a logical and linear process starting from a definite question. Moreover, Anfinsen could not have envisaged that his discovery about protein folding would help understand cystic fibrosis, Alzheimer's disease, and mad cow disease; the significance of a scientific discovery is often not obvious at the start.

In 1989, Anfinsen commented that his own work in 1955 represents a "beautiful example of how an entirely acceptable conclusion can be reached that is entirely wrong because of the paucity of knowledge at that particular time" (Anfinsen, 1989). In his 1955 paper (Anfinsen et al., 1955), Anfinsen concluded that an ordered shape of a protein is not needed for its catalytic function. This is a wrong but reasonable conclusion at the time because protein sequencing and crystallography were not available. Instead of regarding it as "fraud" in science, Anfinsen argued that the advance of science requires "ongoing refinement of data . . . and reinterpretation of formerly held 'truth'." He himself had demonstrated this self-correcting nature of science by spending "the following 15 years or so completely disproving the conclusions." Even Anfinsen' s Nobel-winning discovery has since been found not completely correct: protein folding is not solely determined by amino acid sequence but modified by small proteins called chaperones in cells (Ellis, 1987). Prion, the infectious protein of mad cow disease, is a kind of chaperone.

For decades, protein folding has been an active field of research in biology, chemistry, biochemistry, computer science, and physics. The mystery of protein folding, however, has not been cracked completely after over 50 years. The intramolecular and intermolecular interactions of a protein during folding are hugely complex, and it is still not possible to predict the exact shape of a protein according to its amino acid sequence. This again shows that scientific inquiry is an ongoing process of problem solving where solving one question will open up many more questions.

Adaptation of Anfinsen's Experiment

To make Anfinsen's protein folding experiment (Anfinsen & Haber, 1961) doable in secondary laboratory, some procedures like spectrophotometry, gel chromatography, and gel filtration are replaced by RNA agar to show the ribonuclease activity and dialysis to remove the denaturants. The design principles of the experiment make reference to a study on refolding human serum albumin (Burton et al., 1989). Apart from materials, another consideration is whether secondary students understand the concepts to understand the experiment. A senior secondary biology student should understand the concepts of gene expression, protein structure and functions, and enzymes, but the concepts of thermodynamics and hydrophobic interactions of the polypeptides seem beyond reach of some students, particularly those not studying advanced chemistry and physics. Therefore, we design a basic version of the experiment that only touches upon protein unfolding and refolding, and a more advanced version that goes further to test for the thermodynamic hypothesis.

○ Protein Folding Investigation

Anfinsen's key experiment in 1961 (Anfinsen & Haber, 1961) was modified into a biology investigation, which had been tried out on 23 secondary students from five schools at a university in Hong Kong. The detailed procedure and preparation of the experiments can be found in Online Supplemental Material.

Question

A protein functions only when it is in a specific three-dimensional shape. What determines the folding of a polypeptide chain into its native, three-dimensional shape?

Hypothesis

1. Protein folding is wholly determined by the amino acid sequence of the polypeptide chain itself.
2. Protein is folded into a specific shape by some instructions other than its amino acid sequence in the cytoplasm, such as tRNA, ribosome, or enzymes.

Prediction

According to hypothesis 1, a fully unfolded protein can spontaneously refold back into its native shape in vitro and regain its catalytic function. This is because the instruction required for correct refolding is already encoded in the amino acid sequence of the polypeptide itself. According to hypothesis 2, however, a fully unfolded protein will not spontaneously refold back into its native shape in vitro because the refolding requires the protein synthesis machinery and other instructions in cytoplasm.

○ Design of the Investigation

The unfolding and refolding of an enzyme ribonuclease (RNase) is studied. The activity of RNase can be shown by RNA agar plate, where a clear zone is produced after RNA is broken down by the RNase. To unfold the RNase, 2-mercaptoethanol (2-ME) and urea are used as denaturants. 2-ME breaks down the four disulfide bonds of RNase, urea disrupts the polar nature of the solvent, and together

they unfold the enzyme completely into a polypeptide chain. This fully unfolded RNase is then allowed to refold after removing the denaturants by dialysis. If the polypeptide chain successfully refolds into its native shape, the RNase will regain its catalytic activity.

Procedure

1. Mix 5 solutions in 5 microfuge tubes, as below:
   a 1 ml RNase (0.5mg/ml) + 0.9 ml sodium acetate buffer to keep the pH unchanged
   b 1 ml RNase (0.5mg/ml) + 0.9 ml denaturants (2-ME + urea) to unfold the enzyme
   c dialyzed solution B (dialysis overnight to remove the denaturants for refolding)
   d boiled 0.1 ml RNase + 0.9 ml buffer (as control)
   e 1 ml buffer + 0.9 ml denaturants (as control)
2. Incubate the solutions in a 50°C water bath for 5 minutes to speed up the reaction.
3. Use a cork borer to make five wells in the RNA agar plate using aseptic techniques.
4. Add 100 µl of each solution into a different well of the agar plate.
5. Cover the agar plate with lid and incubate at 37°C for 90 minutes.
6. Overlay the agar plate with 3 ml of 1M perchloric acid for 5 minutes at room temperature. The perchloric acid will make the RNA agar milky.
7. Pour off the perchloric acid to check whether clear zones are formed around the wells. Measure and compare the diameter of the clear zones.

○ Results

Results are shown in Figure 3.

○ Discussion and Analysis

The clear zone at A (Figure 3) indicates that RNase had diffused and broken down the RNA around the well. The breakdown of RNA is not due to the buffer and denaturants, as shown by the absence of clear zone at E. At B, no clear zone has formed, showing that RNase had been denatured by 2-ME and urea by being unfolded into polypeptide chains. At C, when the denaturants had been removed by dialysis, a smaller clear zone was observed. This shows that some RNase molecules had regained catalytic activity by refolding back into its native, three-dimensional shape spontaneously. The clear zone, however, is smaller than that at A because of two probable reasons: (1) Some RNase molecules may have reacted with urea during heating by a process called carbamylation, which prevented their

	Solutions added into the wells	Diameter of clear zone on RNA agar (mm)					Average (mm)
A	RNase + buffer	17	16	16	17	15	16.2
B	RNase + denaturants	11	10	10	10	11	10.4
C	B after dialysis	13	12	13	13	12	12.6
D	Boiled RNase + buffer	17	15	16	16	15	15.8
E	Buffer + denaturants	NA	NA	NA	NA	NA	NA

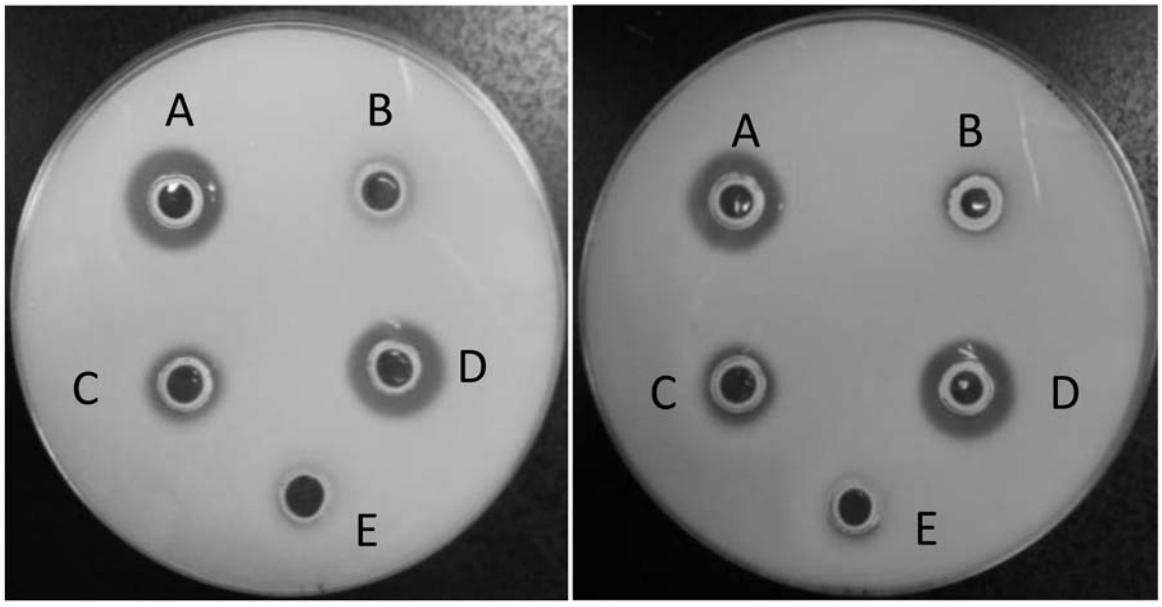

Figure 3. RNA agar plates after incubation and addition of perchloric acid. Clear zones indicate RNase activity.

refolding into native shape. (2) The RNase solution was diluted during dialysis by the water entering the dialyzer by osmosis. The clear zones would become larger and the results more obvious with a longer incubation time. The clear zone at D is as large as that at A, indicating that boiling had no effect on the activity of RNase because its four disulfide bonds strongly hold the conformation of the molecule.

Conclusion

The enzyme RNase can spontaneously refold into its native, three-dimensional shape after being fully unfolded in vitro, which is consistent with the prediction derived from hypothesis 1. Since the unfolded RNase has lost all the information of its shape imparted from the process of protein synthesis, while the only information left is the amino acid sequence, we can conclude that the folding of RNase is determined by its amino acid sequence.

Thermodynamic Hypothesis

The above investigation only shows that protein can refold into its native shape spontaneously, but it offers no explanation for that. For AP or other advanced biology courses, further investigation can be conducted to test the thermodynamic hypothesis of Anfinsen. In AP biology, students are required to know "the specific order of amino acids in a polypeptide (primary structure) interacts with the environment to determine the overall shape of the protein . . . chemical properties (hydrophobic, hydrophilic and ionic), and the interactions of these R groups determine structure and function of that region of the protein" (College Board, 2015, 4.A.1.a.2). The thermodynamic hypothesis stipulates that the native shape of a protein is dependent on the interactions of the R groups with the environment to reach the thermodynamically lowest energy state. This state will change when the external environment changes, such as in the presence of urea. This can be tested by placing the dialyzer in urea solution so that only 2-ME is removed. When the unfolded RNase refolds in the presence of urea, it will refold into many "scrambled" proteins as a result of the randomly formed disulfide bonds. These scrambled molecules cannot refold into native shape even after urea is removed. However, when some 2-ME is added to break the randomly formed disulfide bonds, the RNase can restore its activity by refolding to native shape (Figure 1).

Instructional Approach

The instructional approach employed is guided inquiry (Buck et al., 2008), where the question is provided and the experimental design is partly done by students under teacher guidance. The experiment assumes students have a basic understanding of DNA, protein structure, protein synthesis, enzymes, and dialysis. Students were first told that they would replicate a Nobel-winning experiment to tackle an important question in biology—how a polypeptide chain folds into three-dimensional shape to become a functional protein. After reviewing the relevant concepts, students in groups of four were asked to design an experiment to test the two hypotheses. Students had no difficulty in understanding how to measure RNase activity with RNA agar since they had done similar experiment with amylase and starch agar. After group discussion, each group presented their designs to the class. Their designs mainly differed on how to do replication and control on the RNA agar plate. All groups had proposed a control with only buffer and denaturants, while two groups mentioned a control with boiled RNase. We did not point out that RNase is heat resistant and deliberately used it as a *discrepant event* to confront their misconception that all enzymes can be denatured by heat. The discussion was guided by the teachers, but no "correct" answers were provided. During the incubation of the agar plates that took 1 to 1.5 hours, students could have a lunch break or write up part of their lab report. After incubation, each group showed its plates to the class with a visualizer and explained the results. The whole experiment was finished within 2.5 hours, excluding the time for incubation. Alternatively, the experiment can be done in two consecutive days: On the first day, students investigate the RNase activity with RNA agar, and then do dialysis themselves to test for the hypotheses. After dialysis overnight, they test the activity of the dialyzed RNase solution on the second day.

In general, most students were excited and engaged by the experiment because they felt that they were conducting a Nobel-winning experiment to discover an important biological principle. Particularly important is that they found a Nobel-winning experiment to be within their grasp, which can bolster their self-confidence in learning science. The openness of the investigation is also key to engaging the students because they are often asked to follow prescribed procedures to verify known facts in ordinary school experiments. Despite the fact that the students are not academically strong and the concept of protein folding is unfamiliar to them, their lab reports show that they understand the experiment well and can explain the results satisfactorily. The overall performance of the students has supported that even complex, college-level experiments, when properly guided and designed, can be conducted in secondary classrooms for learning important biological principles and science practices.

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