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Laboratory Experiment

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Analyzing Exonuclease-Induced Hyperchromicity by UV Spectroscopy: An Undergraduate Biochemistry Laboratory Experiment

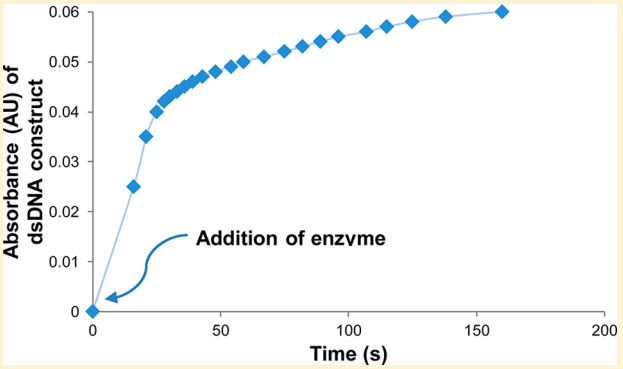
Megan M. Ackerman,[†] Christopher Ricciardi,[†] David Weiss,[†] Alan Chant,[‡] and Christina M. Kraemer-Chant*[,†]

[†]Department of Chemistry, Saint Michael's College, Colchester, Vermont 05439, United States

[‡]Department of Chemistry, University of Vermont, Burlington, Vermont 05405, United States

Supporting Information

ABSTRACT: An undergraduate biochemistry laboratory experiment is described that utilizes free online bioinformatics tools along with readily available exonucleases to study the effects of base stacking and hydrogen bonding on the UV absorbance of DNA samples. UV absorbance of double-stranded DNA at the λ_{max} is decreased when the DNA bases are involved in hydrogen bonding and formation of secondary structure. When an exonuclease is added to the solution containing the DNA, the strand is digested and the interactions disappear, leading to an increase in the absorbance called hyperchromicity. This experiment utilizes exonuclease digestion of DNA to show students how base interactions and secondary structure can alter the spectroscopic properties of a sample and, by extension, how apparent concentration as calculated with Beer–Lambert's law is not necessarily representative of the true concentration of DNA in solution. Teaching applications of this laboratory experiment include enzyme kinetics and activity, secondary structure of single-stranded DNA (and its parallel to RNA structure), and an introduction to bioinformatics.



KEYWORDS: *Upper-Division Undergraduate, Biochemistry, Laboratory Instruction, Hands-On Learning/Manipulatives, Enzymes, Instrumental Methods, Nucleic Acids/DNA/RNA*

INTRODUCTION

In a research setting, DNA oligonucleotides are used in several ways, including for PCR amplification and to study protein–DNA interactions. In many of these applications, knowing the precise concentration of DNA will increase the level of accuracy of the data. Concentration of DNA can be calculated from the absorbance; however, this gives the *apparent* concentration of DNA in solution, which is not always the same as the *true* concentration. This apparent difference is due to either the hypochromic or hyperchromic effect.

The hypochromic effect has been studied for over 50 years (for example, see Cantor and Tinoco's work studying hypochromicity of trinucleoside diphosphates using UV–visible spectroscopy and optical rotatory dispersion[1]) and is observed when DNA bases become stacked. This effect can be induced through the addition of compounds such as drugs comprised of aromatic chromophores, which bind to the DNA through intercalation.[2] This drug:DNA binding leads to a stacking interaction between the drug and the aromatic rings of the base pair of the DNA. The extent of the hypochromic effect is related to the strength of the interaction and the bases involved in that interaction.[3,4] It has been described as the effects upon the UV spectrum of DNA upon contraction of the DNA in the helix axis, sometimes caused by specific cations binding to the phosphate groups of the DNA backbone.[5,6]

The hyperchromic effect is the increase in absorbance at or close to 260 nm upon denaturation of the DNA strand. Hyperchromicity occurs when the DNA is involved in an electrostatic interaction, either with a drug or other molecule or with another DNA strand (or, in the case of single-stranded DNA, with itself through intrastrand interactions). DNA exhibits hydrogen bonding through the familiar complementary (A-T and C-G) base pairing. The bases also exhibit stacking interactions and the hydrophobic effect. When the bases are involved in hydrogen bonding, the aromaticity of the rings is altered and the resonances of the bases become limited. This decreases the UV absorbance of the DNA, which leads to a lower apparent calculated concentration than is actually present in solution. When the DNA is denatured and the hydrogen bonds between complementary bases are disrupted through the

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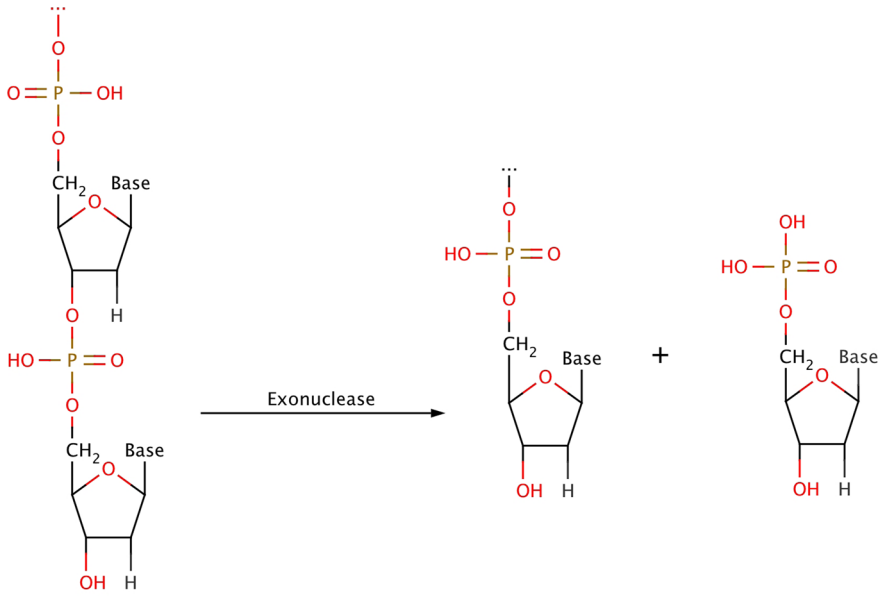

Figure 1. Generic exonuclease activity on a DNA molecule.

use of heat, denaturing agents, pH, or (as described in this work) enzymatic digestion, the base−base interaction is reduced and the UV absorbance of the DNA will increase. This increase can be quite significant depending on the sequence and length of the DNA strand; literature values state that it can be as high as 20−23% for trinucleoside diphosphates.[1] This effect may also be seen in combination with a slight blue shift[7] and has been described as the change in the UV spectrum of DNA resulting from damage of the DNA double-helix structure as well as change in the conformation of the DNA structure.[5,6]

Students do not always appreciate the shortcomings of using UV absorbance directly in calculating concentrations. Many laboratories that require accurate concentrations of DNA either order synthetic DNA oligomers whose concentrations are known with high accuracy or adjust for concentration error by denaturing or digesting the DNA and using the calculated denatured or digested DNA concentration as the true concentration. Nuclear magnetic resonance (NMR) spectroscopy can also be used to determine concentration of biomolecules to a high degree of certainty (typically ∼1%).[8]

The laboratory experiment described herein uses two exonucleases to digest single- and double-stranded DNA. Exonuclease I cleaves single-stranded DNA in a 3′ → 5′ direction, releasing 5′-mononucleotides.[9] T7 exonuclease cleaves double-stranded DNA in a 5′ → 3′ direction, removing 5′-mononucleotides from the 5′ termini or at gaps and nicks of double-stranded DNA.[10,11] A generic exonuclease reaction is shown in Figure 1.

Because exonucleases affect the structure of the DNA, the addition of an exonuclease causes a change in the UV absorption of the DNA sample as described above. This change is considered to be directly representative of how much the hyperchromicity of the system alters the apparent (calculated) concentration of the DNA in solution. (It is important to note again that, while the *true* concentration of the DNA in solution obviously does not change greatly upon addition of 1−2 μL of enzyme in a final volume of 400 μL, the *apparent* concentration as calculated using Beer−Lambert's law does change due to the hyperchromic effect.)

This work describes the development of an upper-division undergraduate level biochemistry laboratory experiment that enables the student to investigate the enzyme-induced hyperchromicity of single- and/or double-stranded polynucleotides. Students gain practical experience in techniques such as the use of micropipettes and quantitative preparation of solutions. They also gain some background in bioinformatics and the folding of biomolecules. Pedagogic goals include increasing student understanding of DNA structure, including single-stranded DNA and RNA secondary structure; bioinformatics tools for predicting DNA folding; enzyme kinetics, specifically focused on exonuclease activity; and overall lab skills, especially the use of micropipettes and UV spectroscopy. The total experiment time is approximately 1.5−2 h and could be performed in an advanced high school class with appropriate revisions based on student background. If desired, the instructor can include how to calculate the molar absorptivity constant for a specific DNA sequence and its use in Beer's law; these are discussed in the Supporting Information.

Related experiments involving determination of rate constants and thermodynamic parameters of DNA systems have been published;[12,13] this experiment is unique in that it is more applicable in a biochemistry lab where understanding of physical chemistry can vary between students.

The student experimental procedure and notes to the instructor are provided in the Supporting Information. The classroom handout issued to the students was written with significant input from the undergraduate students involved in developing this laboratory experiment. All data shown in Figures 2 and 7 were obtained by Biochemistry I students; data shown in Figures 3−6 were obtained by several undergraduate summer research students working on the development of the laboratory experiment and are included as representative data from experiments performed under different conditions. These idealized (student researcher) data indicate how results can change when the lab experiment is run using different concentrations of DNA or enzyme.

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Table 1. DNA Constructs Used in This Experiment

DNA Sequence (5′→3′)	Name	Single- or Double-Stranded System
GCTGAACATATGAATCAG	SSHyp	ssDNA
GACTGACATGTACGC	HypTOP	dsDNA strand
GCGTACATGTCAGTC	HypTOP-r	dsDNA complementary strand

MATERIALS AND METHODS

Equipment

UV studies were performed on an Agilent Technologies UV−visible spectrophotometer. The kinetics analysis software packaged with the UV−vis spectrophotometer was used to obtain readings every 5 s until an upper (digested) baseline was established—typically about 10−15 min. Several of the spectrophotometers used in lab had two types of light sources (the tungsten lamp for visible region analysis and the deuterium lamp for the ultraviolet region). If this was the case, the tungsten lamp was turned off using the system software because of potential signal instability around 260−265 nm.[14]

Initial results were obtained using a 3 mL quartz cuvette; later results were obtained using a 700 μL quartz spectrophotometric microcuvette to reduce the required volumes used. The procedure described herein and in the student handout uses the smaller volume cuvette. These cuvettes were used only for this laboratory experiment and were cleaned thoroughly after each use.

The sources of the cuvettes and information on the instrument used can be found in the Supporting Information.

Chemicals

The sources of the chemicals and DNA can be found in the Supporting Information.

The DNA sequences used herein are listed in Table 1. SSHyp was designed as a single-stranded DNA system that is predicted to form a hairpin loop. HypTOP is the top strand of a double-stranded DNA system. HypTOP-r is the complementary strand of HypTOP.

The SSHyp oligonucleotide was resuspended in the 10× Exonuclease I buffer supplied with the Exonuclease I enzyme (to a final concentration of 100 μM) and used as is. HypTOP and HypTOP-r were resuspended in 10× New England Biolabs NEBuffer 4 to a final concentration of 100 μM. Half of the total volume of each of the HypTOP and HypTOP-r strands was removed, placed in another eppendorf, and annealed by incubation at 70 °C for 10 min using a hot water bath followed by gradual cooling to room temperature on the bench.

Online Tools

Two free online bioinformatics tools were used to allow students to determine if any intramolecular folds are significantly populated at room temperature. These include RNAstructure[15,16] and UNAFold.[17,18] RNAstructure outputs the lowest free-energy structure and a set of low free-energy structures for a DNA sequence. UNAFold also outputs the lowest free-energy structure but includes thermodynamic parameters such as T_m and ΔG.

EXPERIMENTAL PROCEDURES

Bioinformatics Prediction of DNA Strand Secondary Structure

The SSHyp sequence was designed to form a predicted hairpin loop with a relatively high T_m and a negative ΔG. The SSHyp sequence was used as input to RNAstructure and UNAFold, and the results were printed out by the students for inclusion in the classroom discussion. The HypTOP and HypTOP-r were selected to be completely complementary to each other but with minimal secondary structure by themselves, to avoid misfolds and populated single-stranded folds instead of the desired double-stranded molecule.

Enzymatic Digestion of Single-Stranded DNA

The spectrophotometer was blanked and the λ_{max} value for the single-stranded DNA was determined for the sample. To observe hyperchromicity, the absorbance at the sample's λ_{max} was recorded as an initial reading before enzymatic digestion. Diluted Exonuclease I (20 U final activity in reactions using 1 μL of enzyme) was added, and a kinetic scan was obtained by following absorbance at the λ_{max}. The kinetics analysis software was used to obtain readings every 5 s until an upper (digested) baseline was established. Decreasing cycle time showed minimal change in results.

Enzymatic Digestion of Double-Stranded DNA

The λ_{max} for the double-stranded annealed DNA was determined, and the kinetic assay was performed as for the single-stranded DNA, except diluted T7 Exonuclease (10 U final activity in reactions using 1 μL of enzyme) was used.

Treatment of the Results

Change in concentration was taken from the absorbance of the first point (established before addition of enzyme) to the y-intercept of the upper (digested) baseline. Percent change in absorbance was calculated by the students. No further calculations were done.

Controls and Experimental Details

Several controls, their results, and details of the experiments may be found in the Supporting Information.

HAZARDS

Due to the final concentrations of each of the chemicals used (67 mM glycine-KOH, 6.7 mM $MgCl_2$, and 10 mM β-mercaptoethanol, pH 9.5 for the single-stranded studies and 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, and 1 mM DTT, pH 7.9 for the double-stranded studies), there are minimal hazards associated with this experiment.

RESULTS AND DISCUSSION

The image for the SSHyp sequence predicted by UNAFold was the same as that provided by RNAstructure (both with standard parameters as supplied by the program); T_m was predicted to be 36.6 °C, and the ΔG was predicted to be −1.23 kcal/mol. The lowest free-energy structure obtained using the RNAstructure program is shown in Figure 2 for the SSHyp sequence. These values are important for the students to determine because they have direct implications for their results for hyperchromicity in the single-stranded sequences and on the topics discussed, such as single-stranded DNA and RNA structures and stability of folds. Neither of the dsDNA strands

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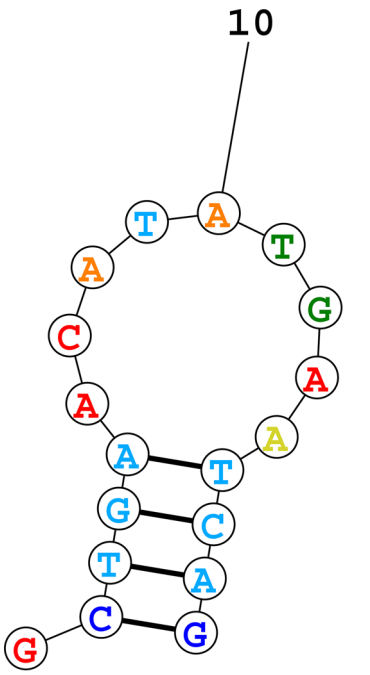

Probability >= 99%
99% > Probability >= 95%
95% > Probability >= 90%
90% > Probability >= 80%
80% > Probability >= 70%
70% > Probability >= 60%
60% > Probability >= 50%
50% > Probability

ENERGY = -1.2 test

Figure 2. Secondary structure with the lowest free energy as predicted by RNAstructure.[15,16] Probability refers to the probability of finding that base in that specific interaction and structure element. Energy is given in kilocalories per mole. All conditions were default as supplied by the site (T = 310.15 K; maximum loop size = 30; maximum %E difference = 10; maximum no. of structures = 10; window size = 3; γ = 1; one iteration; minimum helix length = 3).

were predicted to populate any stable folds at or near room temperature (data not shown).

The first step was to determine appropriate conditions for the experiment to be run by students. The data shown in Figures 3–6 were obtained by undergraduate research students (and are therefore considered idealized) but are representative of student results, some of which are shown in Figure 7. They are included in this work to show predicted results of trials where different concentrations of DNA or volumes of enzyme were used and can be distributed to students in the course if lab time or funds are limited.

Curiously, the single-stranded DNA sample had a λ_{max} at ~277 nm, although shorter wavelengths (255 and 264 nm) were used in several trials as well. For the double-stranded DNA system, the λ_{max} was identified between 250 and 265 nm. In all cases, there was a rapid increase in absorbance after addition of the enzyme.

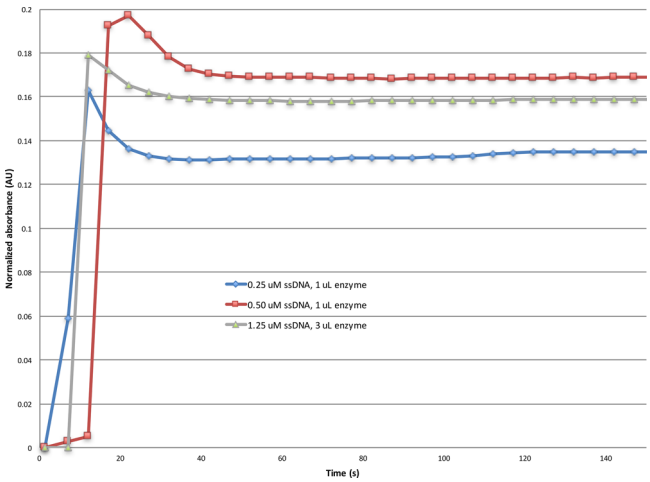

Figure 3. Single-stranded DNA hyperchromicity in 0.25 and 0.50 μM DNA samples (both with 1 μL of enzyme) and 1.25 μM DNA/(3 μL of enzyme). The x-axis has been truncated from 600 to 150 s in this figure and in Figures 4–6 for clarity of results.

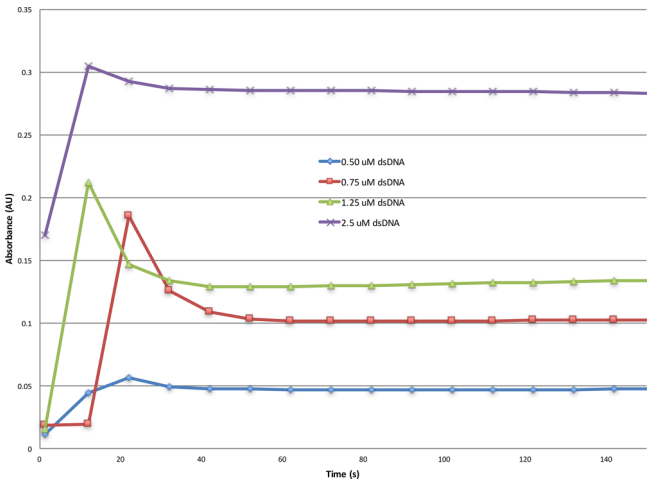

Figure 4. Double-stranded DNA hyperchromicity in various concentrations of DNA (all with 1 μL of enzyme in a final volume of 400 μL).

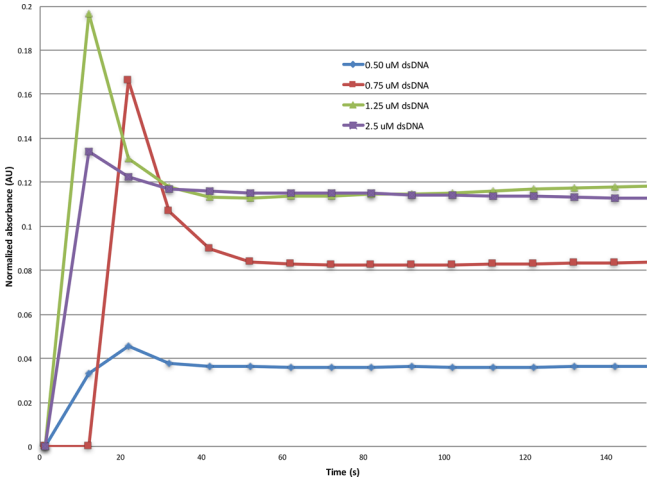

Figure 5. Normalized data from Figure 4, where the absorbance values have been adjusted so that each starting point corresponds to a 0 AU reading.

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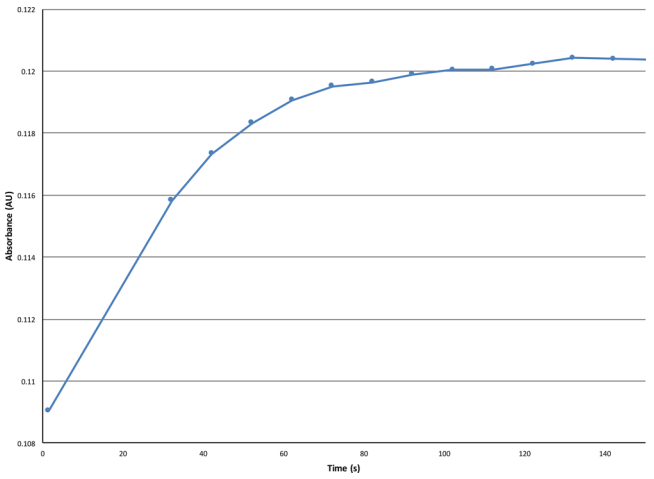

Figure 6. Idealized data showing hyperchromicity of 2.0 μM of double-stranded DNA and 1 μL of Exonuclease I. The data have not been normalized.

To determine the extent of change in absorbance due to DNA structure and binding interactions, the absorbance at the λ_{max} of the undigested single-stranded DNA sample (SSHyp) was monitored after the addition of the appropriate enzyme as a function of time. A 0.25 μM amount of SSHyp and 1 μL of Exonuclease I in 400 μL total volume showed an approximate 75−100% increase in absorbance after addition; this increase was repeatable (n > 5), although experimental conditions, choice of UV−vis spectrophotometer, and the student performing the experiment, etc., did alter the results somewhat. Representative idealized results from three separate trials are shown in Figure 3. These results have been normalized so that AU = 0 before enzyme is added. Note that the spike in Figures 3−5 is due most likely to addition of enzyme and any air bubbles that were introduced; hyperchromicity was determined from the point(s) before enzyme addition to where the upper baseline stabilizes.

As was the case with the single-stranded DNA, the double-stranded DNA sample showed a rapid, significant increase in absorbance after addition of the enzyme. This qualitative increase in absorbance was repeatable (n > 25) and exhibited a dependence on the concentration of DNA used; for example, concentrations between 0.25 and 2.5 μM in a total volume of 400 μL were analyzed, with 1 and 3 μL of T7 Exonuclease, and the change in absorbance ranged from 10 to >500%. Care should be taken in analyzing these results, however; as was the case with the SSHyp results, experimental conditions, choice of UV−vis spectrophotometer, and the student performing the experiment, etc., did alter the results and make this more of a qualitative analysis. No statistical analysis was performed as a result. Representative idealized results from four trials are shown in Figure 4. These results were not normalized in order to show the differences in absorbance between the samples before enzyme is added.

Normalized idealized results for the trials shown in Figure 4, where predigested absorbance was set to 0 and later points were adjusted accordingly for each trial, are shown in Figure 5. The concentration of the annealed DNA or the volume of the enzyme was increased to determine the concentration-dependent effects on the hyperchromicity. Altering the concentration of DNA or volume of enzyme used did qualitatively affect the hyperchromicity results and could be done as part of the lab experiment if time allows. If desired, other changes to the procedure could be made, such as increasing or decreasing temperature, changing pH or salt concentration, and altering the buffer.

As is shown in Figure 6, for 1 μL of the enzyme and 2.0 μM DNA, a transition between undigested and digested DNA was again observed. Here the spike seen due to addition of enzyme and potential introduction of air bubbles was removed in order to show the hyperchromicity in the DNA sample after enzyme digestion took place.

Biochemistry I student results are shown in Figure 7. As can be seen, all showed hyperchromicity as demonstrated by the idealized data in the above figures, although the actual change in absorbance did vary somewhat (hence the qualitative nature of the lab itself). Two of the students normalized their data as directed, and one did not; this provides some comparison between normalized and non-normalized data.

SUMMARY

The student population in our upper-division undergraduate biochemistry laboratories consists of biology, biochemistry, chemistry, and other science majors. All students have had general chemistry and organic chemistry; however, knowledge of biology, molecular biology, and physical chemistry, along with overall lab skills, tend to vary greatly.

The bioinformatics portion has been integrated into two separate semesters of Biochemistry I; one semester involved 50 students individually, and the second semester involved 14 students working in pairs. The background information had been presented in lecture and reviewed in lab, and the first 20−30 min of lab were devoted to using the online tools. These

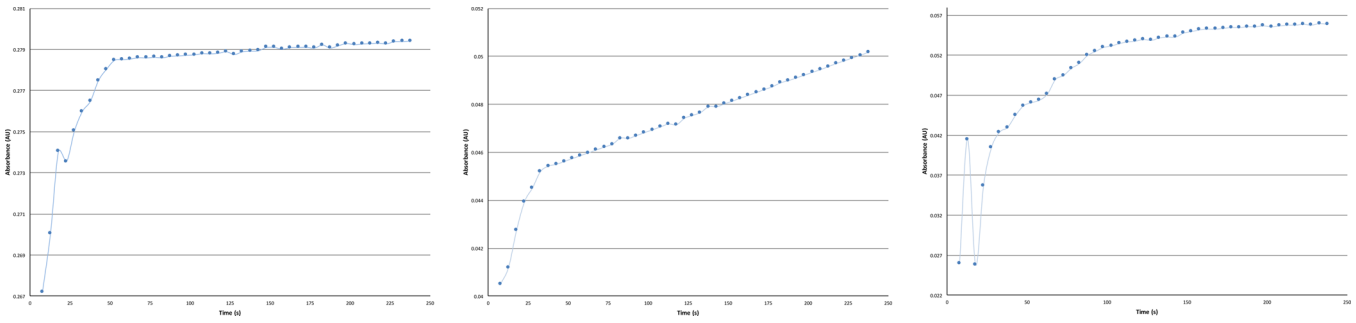

Figure 7. Three graphs of Biochemistry I student results. All samples include 0.75 μM of double-stranded annealed DNA and 1 μL of T7 exonuclease. The leftmost graph includes data that were not normalized, whereas the middle and rightmost graphs include data that were normalized so that AU = 0 before addition of enzyme.

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online software packages allowed them to make predictions about whether a single-stranded DNA molecule will exhibit hyperchromicity upon exonuclease activity. They also allowed the instructor to more deeply discuss thermodynamic parameters as they relate to biochemistry. The main teaching point was how certain single-stranded DNA molecules can exhibit secondary structure without its complementary strand present, and what the importance of this is, such as for PCR amplification or RNA folding.

The experimental procedure was performed on two separate occasions—once by six undergraduate students outside of the classroom to test whether or not the experiment yielded useful results and once as a laboratory experiment in our Biochemistry I curriculum by 14 students working in pairs. As the professor who ran the experiment noted, the classroom handout in the Supporting Information can be adjusted and the idealized data provided herein given to the students if supplies are limited or other conditions such as temperature are to be altered.

The change in absorbance is obvious and repeatable and can range from 15% to >500% based upon the concentration of DNA and enzyme, the student running the experiment, the sequence of the DNA, whether or not it is in solution with its complementary partner, and so forth. The most common issue that arose was poor quantitative skills, including not mixing the solutions adequately upon enzyme addition. This experiment is therefore useful not just to analyze how DNA structure can affect the apparent concentration of the DNA in solution but also to introduce the importance of good analytical skills. Assessment of the achievement of pedagogic goals as described in more detail in the Introduction was done by analyzing student answers to the prelab questions; grading the student notebooks for discussion on the ssDNA structure and reasons for observed hyperchromicity; and determining whether the students observed the hyperchromic shift upon enzyme addition.

Because of the nature of this experiment and the target student population, this is a more qualitative approach to the hyperchromic effect; the absorbance changes were used mainly to discuss how secondary structure in this sequence of DNA led to a change in absorbance and how that would change the calculated concentration of the samples. This method has the benefits of being quick and straightforward, and uses equipment found in almost every biochemistry lab. The experiments could be performed with any size cuvette (although a smaller cuvette is desirable because of the smaller volumes required for analysis) with appropriate changes. There are minimal safety hazards, and the techniques are standard biochemistry lab techniques that upper-division undergraduate students should be able to perform.

This laboratory experiment is an excellent way to allow students to study qualitatively and quickly the effects of secondary structure on an observable phenomenon and could be extended as desired to analyze the kinetics of the selected exonucleases and the effect of single-stranded DNA structure on the efficacy of enzyme digestion, among other topics. If quantitative analysis is desired, other methods such as NMR[8] may be more appropriate in an upper-division undergraduate lab.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.6b00095.

Information on calculating theoretical extinction coefficients, descriptions of controls and their results, the classroom handout (with answers to the prelab questions), and notes to the instructor (PDF, DOCX)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: cchant@smcvt.edu.

Notes

The authors declare no competing financial interest.

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