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Spectrophotometric methods for the study of eukaryotic glycogen metabolism

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Corresponding Author:	Wayne Wilson Des Moines University Des Moines, IA UNITED STATES
Corresponding Author's Institution:	Des Moines University
Corresponding Author E-Mail:	wwilson@dmu.edu
Order of Authors:	Wayne Wilson
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TITLE:

Spectrophotometric Methods for the Study of Eukaryotic Glycogen Metabolism

AUTHORS AND AFFILIATIONS:

Wayne. A. Wilson

Department of Biochemistry & Nutrition, Des Moines University, Des Moines, IA 50312, USA

Email Address of Author:

Wayne. A. Wilson (wayne.wilson@dmu.edu)

SUMMARY:

Techniques to measure the activity of key enzymes of glycogen metabolism are presented, using a simple spectrophotometer operating in the visible range.

ABSTRACT:

Glycogen is synthesized as a storage form of glucose by a wide array of organisms, ranging from bacteria to animals. The molecule comprises linear chains of α1,4-linked glucose residues with branches introduced through the addition of α1,6-linkages. Understanding how the synthesis and degradation of glycogen are regulated and how glycogen attains its characteristic branched structure requires the study of the enzymes of glycogen storage. However, the methods most commonly used to study these enzyme activities typically employ reagents or techniques that are not available to all investigators. Here, we discuss a battery of procedures that are technically simple, cost-effective, and yet still capable of providing valuable insight into the control of glycogen storage. The techniques require access to a spectrophotometer, operating in the range of 330 to 800 nm, and are described assuming that the users will employ disposable, plastic cuvettes. However, the procedures are readily scalable and can be modified for use in a microplate reader, allowing highly parallel analysis.

INTRODUCTION:

Glycogen is widely distributed in nature, with the compound being found in bacteria, many protists, fungi, and animals. In microorganisms, glycogen is important for cell survival when nutrients are limiting and, in higher organisms such as mammals, synthesis and degradation of glycogen serve to buffer blood glucose levels[1–3]. The study of glycogen metabolism is, therefore, of importance to such diverse fields as microbiology and mammalian physiology. Understanding glycogen metabolism requires studying the key enzymes of glycogen synthesis (glycogen synthase and the branching enzyme) and glycogen degradation (glycogen phosphorylase and debranching enzyme). The gold standard assays of glycogen synthase, phosphorylase, branching, and debranching enzyme activities employ radioactive isotopes. For example, glycogen synthase is generally measured in a stopped radiochemical assay by following the incorporation of glucose from UDP-[^{14}C]glucose (in the case of animal and fungal enzymes) or ADP-[^{14}C]glucose (in the case of bacterial enzymes) into glycogen[4,5]. Similarly, glycogen phosphorylase is measured in the direction of glycogen synthesis, following the incorporation of glucose from [^{14}C]glucose-1-phosphate into glycogen[6]. The branching enzyme is assayed by measuring the ability of this

enzyme to stimulate the incorporation of [^{14}C]glucose from glucose-1-phosphate into α1,4-linked chains by glycogen phosphorylase[7], and debranching enzyme activity is determined by following the ability of the enzyme to incorporate [^{14}C]glucose into glycogen[8]. While very sensitive, allowing their use in crude cell extracts with low levels of enzyme activity, the radioactive substrates are expensive and subject to the regulatory requirements attendant to radioisotope use. These barriers place the use of certain assays out of the reach of many workers. However, over the course of many years, an impressive variety of spectrophotometric approaches to the measurement of these enzymes have been described. In general, these approaches ultimately rely upon measuring the production or consumption of NADH/NADPH, or the generation of colored complexes between glycogen and iodine. Thus, they are straightforward and can be carried out using simple spectrophotometers equipped with only tungsten or xenon flash lamps.

Spectrophotometric assays of glycogen synthase rely upon measuring the nucleoside diphosphate released from the sugar nucleotide donor as glucose is added to the growing glycogen chain[9,10]. The procedure for measuring glycogen synthase activity described in section 1 of the protocol, below, is a modification of that outlined by Wayllace et al.[11], and the coupling scheme is shown below:

$(Glucose)_n$ + UPD-glucose → $(Glucose)_{n+1}$ + UDP
UDP + ATP → ADP + UTP
ADP + phosphoenolpyruvate → pyruvate + ATP
Pyruvate + NADH + H^+ → Lactate + NAD^+

Glycogen synthase adds glucose from UDP-glucose onto glycogen. The UDP generated in this process is converted to UTP by nucleoside diphosphate kinase (NDP kinase), in a reaction that generates ADP. The ADP, in turn, then serves as a substrate for pyruvate kinase, which phosphorylates the ADP using phosphoenolpyruvate as a phosphate donor. The resulting pyruvate is converted to lactate by the enzyme lactate dehydrogenase in a reaction that consumes NADH. The assay can, therefore, be performed in a continuous fashion, monitoring the decrease in absorbance at 340 nm as NADH is consumed. It is readily adapted for use with enzymes that require ADP-glucose as a glucose donor. Here, the coupling steps are simpler since the ADP released by the action of glycogen synthase is directly acted upon by pyruvate kinase.

There are a variety of spectrophotometric assays available for the determination of glycogen phosphorylase activity. In the classical version, the enzyme is driven backward, in the direction of glycogen synthesis, as shown below:

$(Glucose)_n$ + Glucose-1-phosphate → $(Glucose)_{n+1}$ + P_i

At timed intervals, aliquots of the reaction mixture are removed, and the amount of phosphate liberated is quantified[12,13]. In our hands, this assay has been of limited use due to the presence of readily measurable free phosphate in many commercial preparations of glucose-1-phosphate, combined with the high concentrations of glucose-1-phosphate required for phosphorylase action. Rather, we have routinely employed an alternative assay that measures the glucose-1-

phosphate released as glycogen is degraded by phosphorylase[13]. A coupled reaction scheme, illustrated below, is employed.

$(Glucose)_n + P_i \rightarrow (Glucose)_{n-1} + Glucose\text{-}1\text{-}phosphate$
$Glucose\text{-}1\text{-}phosphate \rightarrow Glucose\text{-}6\text{-}phosphate$
$Glucose\text{-}6\text{-}phosphate + NADP^+ \rightarrow 6\text{-}phosphogluconolactone + NADPH + H^+$

The glucose-1-phosphate is converted into glucose-6-phosphate by phosphoglucomutase, and the glucose-6-phosphate is then oxidized to 6-phosphogluconolactone, with the concomitant reduction of $NADP^+$ to NADPH. The procedure detailed in section 2 of the protocol, below, is derived from methods described by Mendicino et al.[14] and Schreiber & Bowling[15]. The assay can be readily performed in a continuous fashion, with the increase in absorbance at 340 nm over time, allowing the determination of the reaction rate.

Spectrophotometric determination of debranching enzyme activity relies upon the measurement of the glucose released by the action of the enzyme on phosphorylase limit dextrin[16]. This compound is made by treating glycogen exhaustively with glycogen phosphorylase. Since glycogen phosphorylase action stops 4 glucose residues away from an α1,6-branch point, the limit dextrin contains glycogen, the outer chains of which have been shortened to ~4 glucose residues. Preparation of phosphorylase limit dextrin is described here, using a procedure derived from those developed by Taylor et al.[17] and Makino & Omichi[18].

Debranching is a two-step process. The 4-α-glucanotransferase activity of the bifunctional debranching enzyme first transfers three glucose residues from the branch point to the nonreducing end of a nearby α1,4-linked glucose chain. The single, α1,6-linked glucose residue remaining at the branch point is then hydrolyzed by the α1,6-glucosidase activity[19]. The assay is typically performed in a stopped fashion, the glucose released after a given time (or series of times) being measured in a coupled enzyme assay as shown below:

$(Glucose)_n \rightarrow (Glucose)_{n-1} + Glucose$
$Glucose + ATP \rightarrow Glucose\text{-}6\text{-}phosphate + ADP$
$Glucose\text{-}6\text{-}phosphate + NADP^+ \rightarrow 6\text{-}phosphogluconolactone + NADPH + H^+$

The determination of NADPH produced gives a measure of glucose production. The procedure outlined in section 3 of the protocol, below, is based upon one described by Nelson et al.[16]. Like the other methods that rely upon the consumption or generation of NADH/NADPH, the assay is quite sensitive. However, the presence of amylases or other glucosidases, which can also liberate free glucose from phosphorylase limit dextrin, will cause significant interference (see Discussion).

The colorimetric determination of branching enzyme activity relies upon the fact that α1,4-linked chains of glucose adopt helical structures that bind to iodine, forming colored complexes[20]. The color of the complex formed depends upon the length of the α1,4-linked chains. Thus, amylose, which consists of long, largely unbranched chains of α1,4-linked glucose forms a deep blue complex with iodine. In contrast, glycogens, the outer chains of which are generally in the order

of only 6 to 8 glucose residues long, form orange-red complexes. If a solution of amylose is incubated with branching enzyme, the introduction of branches into the amylose results in the generation of shorter α1,4-linked glucose chains. Thus, the absorption maximum of the amylose/iodine complexes shifts toward shorter wavelengths. The procedure discussed here is derived from that detailed by Boyer & Preiss[21] and branching enzyme activity is quantified as a reduction in absorption of the amylose/iodine complex at 660 nm over time.

As should be readily apparent from the discussion above, the fact that the color of the complexes formed between iodine and α1,4-glucose chains varies with the chain length, which means that the absorbance spectra of glycogen/iodine complexes should vary with the degree of glycogen branching. This is indeed the case, and less-branched glycogens/glycogens with longer outer chains absorb light at a longer wavelength than glycogens that are more branched/have shorter outer chains. The iodine staining reaction can therefore be used to gain rapid, qualitative data on the degree of glycogen branching[22]. The orange-brown color forms when glycogen complexes with iodine is not particularly intense. However, color development can be enhanced by the inclusion of saturated calcium chloride solution[22]. This boosts the sensitivity of the method some 10-fold and allows ready analysis of microgram quantities of glycogen. The assay for the determination of branching described in section 4 of the protocol, below, is adapted from a procedure developed by Krisman[22]. It is conducted simply by combining the glycogen sample with iodine solution and calcium chloride in a cuvette and collecting the absorption spectrum from 300 nm to 800 nm. The absorbance maximum shifts toward longer wavelengths as the degree of branching decreases.

Collectively, the methods described here provide simple, reliable means of assessing the activities of the key enzymes of glycogen metabolism, and for obtaining qualitative data on the extent of glycogen branching.

PROTOCOL:

1. Determination of glycogen synthase activity

1.1. Prepare stock solutions of required reagents as indicated in **Table 1** (prior to the experimental day).

[Place **Table 1** here]

1.2. On the day of the assay, prepare a fresh working solution of 4 mM NADH by dissolving 4.5 mg of NADH in 1.5 mL of 50 mM Tris-HCl, pH 8.0. Store on ice, protected from the light.

1.3. Thaw stock solutions of UDP-glucose, ATP, phosphoenolpyruvate, and NDP kinase on ice.

1.4. Pre-heat a water bath to 30 °C.

1.5. Set up each glycogen synthase assay in a 1.5 mL tube by adding the reaction mixture reagents listed in **Table 2**.

[Place **Table 2** here]

NOTE: To facilitate the set-up, a master mix can be made containing enough of each of the above-listed reagents to complete the number of assays planned.

1.6. Prepare a blank reaction, where the NADH in the above mixture is replaced with water. Transfer to a disposable methacrylate cuvette and use this to set the zero on the spectrophotometer at 340 nm.

1.7. Take one 770 µL of the aliquot of reaction mixture in a 1.5 mL tube. Add 2 µL of NDP kinase and 2 µL of pyruvate kinase/lactate dehydrogenase mixture, mix gently, and incubate at 30 °C for 3 min to pre-warm the reaction mixture.

1.8. Add 30 µL of the sample containing glycogen synthase in 20 mM Tris buffer, pH 7.8; mix, and transfer the reaction mixture to a disposable methacrylate cuvette.

1.9. Place the cuvette into the spectrophotometer and record the absorbance at 340 nm at timed intervals for 10 to 20 min. Plot the absorbances obtained against time.

NOTE: A reaction in which the glycogen synthase sample is replaced with 20 mM Tris buffer should be conducted to control for non-enzymatic oxidation of NADH. Depending upon the purity of the sample, other control reactions may be required. See Discussion for details.

1.10. Determine the reaction rate (see **Results** for details).

2. Determination of glycogen phosphorylase activity

2.1. Prepare stock solutions as indicated in **Table 3** (prior to experimental day).

[Place **Table 3** here].

2.3. Pre-heat a water bath to 30 °C

2.4. Set up each glycogen phosphorylase assay in a 1.5 mL tube by adding the reaction mixture reagents listed below (**Table 4**).

[Place **Table 4** here].

NOTE: To facilitate the set-up, a master mix can be made containing enough of each of the above-listed reagents to complete the number of assays planned.

2.5. Prepare a blank reaction containing the components listed in **Table 4** but add an additional 30 µL of 25 mM PIPES buffer, pH 6.8 (prepared by diluting 125 mM PIPES buffer 1/5 with water). Transfer to a disposable methacrylate cuvette and use to set the zero on the spectrophotometer at 340 nm.

2.6. Take one 770 µL aliquot of reaction mixture in a 1.5 mL tube. Add 1 µL of glucose-6-phosphate dehydrogenase and 1 µL of phosphoglucomutase, mix gently, and incubate at 30 °C for 3 min to pre-warm the reaction mixture.

2.7. Add 30 µL of the sample containing glycogen phosphorylase in 25 mM PIPES buffer, pH 6.8. Mix and transfer the reaction mixture to a disposable methacrylate cuvette.

2.8. Place the cuvette into the spectrophotometer and record the absorbance at 340 nm at timed intervals for 10 to 20 min. Plot the absorbances obtained against time.

NOTE: A reaction in which glycogen phosphorylase is replaced with 25 mM PIPES buffer should be included. Depending upon the purity of the glycogen phosphorylase sample, other controls may also be needed (see Discussion for details).

2.9. Determine the reaction rate (see Representative Results for details).

3. Determination of glycogen debranching enzyme activity

3.1. Prepare stock solutions as indicated in **Table 5** (prior to the experimental day).

[Place **Table 5** here].

3.2 Prepare phosphorylase limit dextrin

3.2.1. Dissolve 0.3 g of oyster glycogen in 10 mL of 50 mM sodium phosphate buffer, pH 6.8.

3.2.2. Dissolve sufficient lyophilized phosphorylase A powder to yield 60 U of activity in 50 mM phosphate buffer, pH 6.8.

NOTE: Depending upon the lot of phosphorylase A purchased, the mass needed will vary but is generally between 5 and 10 mg of powder.

3.3. Add 60 U of phosphorylase A to the glycogen solution and transfer to a dialysis bag. Dialyze at room temperature against 1 L of 50 mM sodium phosphate buffer, pH 6.8 for 8 h. Change to the fresh dialysis buffer and continue the incubation overnight.

3.4. Add another 10 U of phosphorylase A and change to fresh dialysis buffer. After 8 h, again change to fresh dialysis buffer and continue the incubation overnight.

3.5. Transfer the contents of the dialysis bag to a centrifuge tube and boil for 10 min. Chill on ice, and then centrifuge at 10,000 x *g* for 15 min.

3.6. Transfer the supernatant to a dialysis bag and dialyze for 8 h against three changes of 2 L of distilled water.

3.7. Transfer the contents of the dialysis bag to a 50 mL centrifuge tube. Measure the volume and add two volumes of ice-cold absolute ethanol to precipitate the limit dextrin. Let the tube stand on ice for 30 min.

NOTE: A white precipitate should begin to form immediately upon the addition of ethanol but, if it does not, add a drop of 3 M NaCl.

3.8. Centrifuge at 15,000 x *g* for 15 min and discard the supernatant. Rinse the white pellet of limit dextrin twice with 66% v/v ethanol, using ~30 mL for each rinse.

3.9. Transfer the limit dextrin to a mortar and allow to air dry completely. When the limit dextrin is dry, grind to a powder with a pestle and transfer to a suitable vessel for storage; dry at 4 °C.

3.10. For use as debranching enzyme substrate, prepare a 1% w/v solution in water.

3.11. Pre-heat a water bath to 30 °C.

3.12. Pre-heat a heating block or water bath to 95 °C.

3.13. Prepare four 1.5 mL tubes each containing 100 µL of maleate buffer, 80 µL of phosphorylase limit dextrin, and 10 µL of water. These tubes will be used to conduct the debranching enzyme assay. Label two of the tubes Reaction and the other two tubes Control.

3.14. At timed intervals, add 10 µL of the debranching enzyme sample to the Reaction tubes and 10 µL of buffer which was used to prepare the branching enzyme sample to the Control tubes. Incubate at 30 °C.

3.15. At defined time points (e.g., 5-, 10-, and 20-min incubation), withdraw 50 µL from each Reaction and Control tube and immediately place into the heating block or water bath at 95 °C. Heat for 3 min.

3.16. Centrifuge at 15,000 x *g* for 2 min in order to remove precipitated protein.

NOTE: At this point, the procedure can be halted if needed. The heated samples can be stored frozen at -20 °C until proceeding to the measurement of released glucose (step 4, below).

3.17. Measurement of released glucose

3.17.1. Transfer 40 µL of the supernatant from the heated samples to disposable methacrylate cuvettes and add 833 µL of triethanolamine hydrochloride/magnesium sulfate buffer, 67 µL of NADP/ATP mix, and 60 µL of water. Mix by pipetting up and down gently, being careful not to introduce air bubbles.

NOTE: To facilitate the set-up, a master mix can be made containing enough of each of the above-listed reagents to complete the number of assays planned.

3.17.2. Prepare a blank reaction by combining 100 µL of maleate buffer, 80 µL of phosphorylase limit dextrin, and 20 µL of water. Mix well, and transfer 40 µL to a disposable methacrylate cuvette. Add 833 µL of triethanolamine hydrochloride/magnesium sulfate, etc. as described in step 3.17.1.

3.17.3. Set the zero on the spectrophotometer at 340 nm using the blank reaction.

3.17.4. Add 0.5 µL of glucose-6-phosphate dehydrogenase to each cuvette. Mix gently by pipetting up and down slowly. Incubate at room temperature for 10 min, and then record the absorbance at 340 nm.

NOTE: The absorption values should be low, signifying little contamination of the samples with glucose-6-phosphate.

3.17.5. Add 0.5 µL of hexokinase to each cuvette. Mix gently by pipetting up and down slowly. Incubate at room temperature for 15 min, and then record the absorbance at 340 nm.

3.17.6. Continue the incubation at room temperature for an additional 5 min. Record the absorbance at 340 nm once again. If the absorption has increased from that recorded at 15 min, continue incubation for a further 5 min and again check the absorption. Record the final absorption at 340 nm obtained.

3.17.7. For each sample, subtract the absorbance at 340 nm recorded after the addition of glucose-6-phosphate dehydrogenase from the final absorbance obtained after addition of hexokinase. Plot the values obtained against the length of time that the corresponding sample had been incubated with debranching enzyme.

4. Determination of glycogen branching enzyme activity

4.1. Prior to the experimental day, prepare iodine/KI solution by first dissolving 2.6 g of KI in 10 mL of water. In a fume hood, weigh out 0.26 g of iodine and add to the KI solution.

CAUTION: Iodine is harmful when in contact with the skin or if inhaled. Mix to dissolve the iodine and store at 4 °C, protected from the light. Also prepare 125 mM PIPES buffer, pH 6.8 (see **Table 3**).

4.2. When beginning experiments, make a working stock of acidified iodine reagent.

4.2.1. Take 45.7 mL of water in a 50 mL tube and add 150 µL of iodine/KI solution followed by 150 µL of 1 M HCl.

4.2.2. Mix well and store at 4 °C, protected from the light. The solution is stable for at least 3 days under these conditions.

4.3. On the day of the experiment, make a fresh 10 mg/mL solution of amylose.

4.3.1. Weigh out 50 mg of amylose and transfer to a 15 mL tube.

4.3.2. Add 200 µL of absolute ethanol and shake gently.

4.3.3. Add 500 µL of 2 M KOH and shake gently.

CAUTION: KOH causes severe skin burns and eye damage. Use appropriate personal protective equipment.

4.3.4. Add 0.5 mL of water while shaking gently. If the amylose does not dissolve completely, add an additional 0.5 mL of water.

4.3.5. Adjust the pH to ~6.5 to 7.0 with 1 M HCl.

CAUTION: HCl may cause eye, skin, and respiratory tract irritation. Use appropriate personal protective equipment.

4.3.6. Add water to reach a final volume of 5 mL.

4.3.7. Sterilize by passage through a 0.2 µm syringe end filter and store at room temperature. Do not chill or freeze.

4.4. Pre-heat a water bath to 30 °C.

4.5. Prepare twelve 1.5 mL tubes, each containing 1 mL of acidified iodine reagent. Set aside on the bench. These will be used to stop the branching enzyme reaction.

4.6. Prepare four 1.5 mL tubes each containing 150 µL of amylose, 150 µL of PIPES buffer, and 45 µL of water. These tubes will be used to conduct the branching enzyme assay. Label two of the tubes Reaction and the other two tubes Control.

4.7. At timed intervals, add 5 µL of branching enzyme sample to the Reaction tubes and 5 µL of buffer which was used to prepare the branching enzyme sample to the Control tubes. Incubate at 30 °C.

4.8. At defined time points (e.g., 5, 10, and 15 min incubation), withdraw 10 µL from each Reaction and Control tube and add to the 1.5 mL tubes that contain 1 mL of acidified iodine reagent. Add an additional 140 µL of water and mix well. Transfer to disposable cuvettes.

NOTE: The samples should be blue in color and the solution should be free of any precipitate. The color formed is stable for at least 2 h at room temperature.

4.9. Prepare a sample that contains 1 mL of acidified iodine reagent and 150 µL of water. Mix well and transfer to a cuvette. Use this cuvette to set the zero on the spectrophotometer at 660 nm.

4.10. Read the absorbance of each of the twelve samples at 660 nm. Determine the rate of the branching enzyme reaction by subtracting the absorbance obtained in the presence of branching enzyme (Reaction) from the absorbance when no branching enzyme is present (Control) at each time point. See Representative Results for details.

5. Qualitative assessment of glycogen branching

5.1. Prepare saturated calcium chloride solution by adding 74.5 g of anhydrous calcium chloride to ~40 mL of water and stirring. Add a little more water and continue to stir. Make the volume up to 100 mL with water and continue stirring until the $CaCl_2$ is fully dissolved.

5.2. Prepare a working stock of iodine/$CaCl_2$ color reagent by mixing 50 µL of KI/iodine stock solution (see step 4.1, above) with 13 mL of saturated $CaCl_2$ solution in a 15 mL tube. Mix well and store at 4 °C, protected from the light. The solution is stable for at least 1 week under these conditions.

5.3. Determination of branching

5.3.1. In a 1.5 mL tube, combine 650 µL of iodine/$CaCl_2$ color reagent stock with 100 µL of water and mix thoroughly. Transfer the solution to a disposable methacrylate cuvette.

NOTE: The solution in the cuvette should be clear and pale yellow in color.

5.3.2. Place in the spectrophotometer and, running in a wavelength scanning mode, collect a background spectrum from 300 nm to 800 nm.

5.3.3. In a 1.5 mL tube, combine 650 µL of working iodine/$CaCl_2$ color reagent with 50 µg of oyster glycogen. Bring the final volume to 750 µL with water and mix thoroughly. Transfer the solution to a disposable methacrylate cuvette.

NOTE: The solution in the cuvette should be clear and a deep orange/brown color.

5.3.4. Place in the spectrophotometer and collect an absorption spectrum from 330 nm to 800 nm.

5.3.5. Repeat steps 4.4.3 through 4.4.4 with 50 µg of amylopectin and 30 µg of amylose.

NOTE: The amylopectin sample should be yellow/green and the amylose sample should be green/blue. Both samples should be clear. The colored complexes formed are stable, with no change in absorption spectrum, for at least 1 h at room temperature.

5.3.6. To obtain an indication of the branched structure of an uncharacterized glycogen sample, combine 25 µg to 50 µg of glycogen with 650 µL of working iodine/$CaCl_2$ color reagent. Proceed as above, bringing the volume to 750 µL with water, mixing thoroughly, and transferring to a methacrylate cuvette.

NOTE: The glycogen sample should yield a yellow/orange to orange/brown color depending upon the degree of branching (length of outer chains) of the glycogen present. Again, the sample should be clear. See **Representative Results** for details.

5.3.7. Collect the absorption spectrum.

REPRESENTATIVE RESULTS:

Determination of glycogen synthase activity

Figure 1 shows representative results from glycogen synthase assays using purified enzymes. In panel A, following a slight lag, there was a linear decrease in the absorption at 340 nm over time for a period of around 12 min. The rate of change in absorption in **Figure 1A** was ~0.12 absorbance units/min. A rate of change in absorbance between ~0.010 and ~0.20 absorbance units/min is optimal and the amount of glycogen synthase added should be adjusted to yield rates within this range. In panel B, the result of adding too much glycogen synthase to the assay is shown. Here, the reaction was complete within the first 2 min. The Control reaction, which in these cases contained no glycogen synthase, showed no measurable decrease in absorbance over time. As elaborated upon in the Discussion, the use of tissue homogenates in this assay is perfectly feasible, although additional Control reactions are required.

The protocol described here uses oyster glycogen as a substrate, which works well with glycogen synthases from many different species. However, it should be noted that glycogen synthases may display quite variable activity depending upon the type of glycogen employed. Therefore, it is advisable to survey a variety of forms of glycogen prior to beginning any detailed study.

The protocol given includes glucose-6-phoposphate in the reaction mixture, since many glycogen synthases are allosterically activated by this compound[9,23–26]. Conducting assays in the presence and absence of glucose-6-phosphate (making up the reaction volume with water), allows

calculation of the -/+ glucose-6-phosphate activity ratio, which is a useful indication of the phosphorylation state of mammalian and fungal glycogen synthases[1,27].

The determination of glycogen synthase activity from the change in absorbance is rather straightforward. The extinction coefficient of NADH is taken as 6220 M^{-1} cm^{-1}, allowing calculation of the rate of change in NADH concentration from the rate of absorbance change as follows:

A rate of change in absorption of 0.12 units/min corresponds to 0.12/6220 = 1.93 x 10^{-5} mol/L/min change in NADH concentration. The volume in the cuvette was 0.8 mL, meaning that the change in amount of NADH was: 1.93 x 10^{-5} x 0.8 x 10^{-3} = 3.46 x 10^{-8} mol/min. The volume of enzyme added was 60 µL, 3.46 x 10^{-8} x (1000 / 60) = 5.76 x 10^{-7} mol NADH consumed/min/mL enzyme.

Since there is a one-to-one relationship between the NADH consumed and the glucose incorporated into glycogen, the rate of reaction can be expressed as 5.76 x 10^{-7} mol glucose incorporated/min/mL.

When the protein content of the enzyme sample is known, the specific enzyme activity can be expressed as µmol glucose incorporate/min/mg protein or nmol glucose incorporated/min/mg protein, as appropriate.

As mentioned in the Introduction, the protocol is easily adapted to measure the activity of glycogen synthases that use ADP-glucose as a glucose donor. This is achieved by the simple substitution of UDP-glucose with ADP-glucose in the reaction mixture. Furthermore, both NDP kinase and ATP are omitted from the reaction mixture, since the ADP that is released during glycogen synthase action is a direct substrate for pyruvate kinase. [Place Figure 1 here].

Determination of glycogen phosphorylase activity

Figure 2 shows representative data from a glycogen phosphorylase assay using purified enzyme. With the preparation used here, the assay was linear for approximately 3 min. The inset shows a regression line drawn through the points from time 0 to 2.5 min. The slope of this line shows the rate of absorbance change to be 0.022 absorbance units/min. A rate of absorbance increase of around 0.01 to 0.04 is optimal, since the assay will deviate from linearity quite rapidly if too much enzyme is present. The rate of NADPH formation is calculated from the extinction coefficient which, like that of NADH, is 6220 M^{-1} cm^{-1}. For every 1 mol of NADPH formed, one mol of glucose-1-phosphate had been produced by the action of glycogen phosphorylase. Enzyme activity can therefore be expressed as the amount of glucose-1-phosphate released from glycogen per unit time, following a calculation similar to that outlined above.

The reaction conditions are readily adaptable for those phosphorylases that are sensitive to allosteric modulation. The requisite effectors are simply included in the reaction master mix, replacing some of the water. An important caveat is that the effector itself must be shown not to

influence the activity of the coupling enzymes, phosphoglucomutase, and glucose-6-phosphate dehydrogenase.

Lastly, as with glycogen synthase described above, the type of glycogen used as the substrate may impact the rate of the reaction. While oyster glycogen works well with phosphorylases from many species, it may not always be the optimal choice. [Place Figure 2 here].

Determination of glycogen debranching enzyme activity

The data shown in **Figure 3** are representative of a glycogen debranching enzyme assay using purified debranching enzyme. At each time point, the change in absorption that occurred in the absence of added branching enzyme (Control) was subtracted from the change in absorption that occurred in the presence of branching enzyme (Reaction). The resulting absorbance values were then plotted. As above, a rate of change of NADPH concentration is calculated from the initial slope of the curve by regression analysis. In this example, the increase in NADPH per unit time was linear for 10 min, with a slope of 0.0079 absorbance units/min. While these data are perfectly useable, the addition of slightly less enzyme would have given a shallower slope and allowed for a longer linear phase. Alternatively, additional readings could be taken by removing aliquots for measurement at 2 min and 7 min incubation. Determination of debranching enzyme activity is very straightforward, since 1 mol of NADPH is formed for every 1 mol of glucose released by the α1,6-glucosidase activity of debranching enzyme. Thus, reaction rate can be expressed as amount of glucose released from phosphorylase limit dextrin per unit time, following the same type of calculation as was used for the glycogen synthase and phosphorylases assays, above. [Place Figure 3 here].

Determination of glycogen branching enzyme activity

Figure 4 shows data from glycogen branching enzyme assays. At each of the indicated time points, the absorbance of the Control and Reaction samples were measured. The absorbance of the Reaction sample at 660 nm was subtracted from that of the corresponding Control sample, and the absorbance difference was plotted against time. A regression line was then drawn through the points (panel A). Reaction rate can be expressed simply as the change in absorbance at 660 nm per unit time. The maximum change in absorbance that can occur in this assay is only ~0.4 absorbance units, representing maximal branching of the added amylose by the branching enzyme (panel B). Furthermore, when the absorbance of the Reaction tubes drops more than ~0.2 absorbance units below that of the Control, the assay is no longer within the linear range and no estimation of reaction rate can be made (panel B).

The amylose used as a substrate in this procedure will begin to leave solution quite readily if chilled or frozen and then thawed. Therefore, it is important to make the amylose substrate solution fresh and to ensure that no precipitate has formed prior to use. [Place Figure 4 here].

Qualitative assessment of the extent of glycogen branching

Amylose, amylopectin, phosphorylase limit dextrin, glycogen isolated from yeast were combined with iodine/saturated calcium chloride solution and the absorption spectra of the resulting complexes were collected (**Figure 5**). Using the masses of glycogen, amylopectin, and amylose

given in the protocol described above, the maximum absorbance reading obtained should be around 0.7 to 0.8, as shown here (panels A and B). The absorbance maxima for amylose and amylopectin are around 660 nm and 500 nm, and 385 nm respectively. Phosphorylase limit dextrin was included here, since collecting the absorbance spectrum of phosphorylase limit dextrin/iodine complexes provides a quick check of the extent of phosphorylase digestion achieved during the preparation of this debranching enzyme substrate. Glycogen from most sources produces two peaks, one at approximately 400 nm and a second peak at 460 nm (**Figure 5B**). Leftward shifts in the absorbance spectra of glycogen indicate increased branching/decreased outer chain length. Conversely, rightward shifts indicate decreased branching/increased outer chain length.

The saturated calcium chloride solution is dense and the added glycogen samples will form a layer across the top when added. Therefore, careful mixing is needed to obtain a homogenous solution. In addition, if the carbohydrate samples used are not fully dissolved before mixing with the calcium chloride solution, dark-staining aggregates will form in the cuvette. These aggregates will obviously impede collection of an absorption spectrum and it is important to ensure that the solution in the cuvette is clear before proceeding with any measurements. [Place Figure 5 here]

FIGURE AND TABLE LEGENDS:

Table 1: Stock solutions required for the assay of glycogen synthase activity.

Table 2: Composition reaction mixture for assay of glycogen synthase activity.

Table 3: Stock solutions required for the assay of glycogen phosphorylase activity.

Table 4: Composition reaction mixture for assay of glycogen phosphorylase activity.

Table 5: Stock solutions required for the assay of glycogen debranching enzyme activity.

Figure 1: Representative results from assays of glycogen synthase activity. The spectrophotometer was set to take one reading per min for a total time of 20 min. Panel A shows the expected short lag phase followed by a linear decrease in absorbance with time (Experimental). There was no decrease in the absorption noted in the Control reaction. Reaction rate is calculated from the slope of the absorbance change in the linear phase (from 5 to 16 min). Panel B shows the result of adding too much enzyme. Here, the NADH is exhausted within 2 min.

Figure 2: Representative results from assays of glycogen phosphorylase activity. The spectrophotometer was set to take one reading every 30 s for a total time of 10 min. There was a steady increase in absorbance recorded in the presence of glycogen phosphorylase (Experimental), while the reaction without added phosphorylase remained at baseline (Control). The inset shows an enlargement of the initial reaction period, demonstrating the linearity of product formation with respect to time.

Figure 3: Representative results from assays of glycogen debranching enzyme activity. Samples of phosphorylase limit dextrin were treated with debranching enzyme for 5, 10, 20, or 40 min. The increase in absorbance at 340 nm, produced as NADP was reduced to NADPH in a coupled enzyme assay, was measured in samples taken at each of these time points. The reaction showed a linear phase, persisting for at least 10 min.

Figure 4: Representative results from assays of glycogen branching enzyme activity. Samples of amylose were treated with branching enzyme. Aliquots were removed at the time points shown and added to an acidified iodine reagent. The absorbance of amylose/iodine complex formed was then measured at 660 nm. The data shown represent the difference in absorbance between control incubations that lacked branching enzyme and reactions that contained branching enzyme. Panel A shows a decrease in absorbance at 660 nm due to debranching enzyme activity, which was linear for ~20 min. Panel B illustrates the narrow dynamic range of the assay, where the maximum change in absorbance that can be produced is ~0.4 absorbance units and linearity is lost when the change in absorption is ~0.2 absorbance units.

Figure 5: Representative results from the qualitative assessment of glycogen branching. Samples of purified phosphorylase limit dextrin, amylopectin, amylose (Panel A) or glycogen (Panel B) were combined with iodine/saturated calcium chloride solution and the absorption spectra of the resulting complexes were measured from 330 nm to 800 nm.

DISCUSSION:

In general, the key advantages of all of the methods presented are their low cost, ease, speed, and lack of reliance upon specialized equipment. The major disadvantage that they all share is sensitivity compared to other available methods. The sensitivity of the procedures that involve production or consumption of NADH/NADPH are easy to estimate. Given that the extinction coefficient of NADH/NADPH is 6.22 M^{-1} cm^{-1}, simple arithmetic indicates that ~10–20 μM changes in concentration can be readily detected. With the assay volumes described in the current article, this corresponds to the ability to measure quantities in the range of ~10–20 nmol. Arguably, this is fairly sensitive. However, it is possible to adjust the specific activity of radiolabeled substrates such that sensitivity can be increased beyond the limit of the spectrophotometric assays quite readily. Although the branching enzyme assay and the qualitative assessment of glycogen branching both rely upon the formation of complexes between iodine and α1,4-linked glucose polymers, the output from each assay is different and their sensitivities are likewise different. Specific considerations for each of the assays described are discussed below.

Determination of glycogen synthase activity

The coupled enzyme assay described here allows for continuous assay of glycogen synthase activity, which is useful in the determination of kinetic parameters. It is also readily scalable and a very similar procedure has been described for use in microtiter plates, allowing for highly parallel measurements of enzyme activity to be made[28]. We routinely use this assay with purified, recombinant glycogen synthases[29]. However, the procedures described were originally developed for use in tissue homogenates (for examples, see Danforth[10] and Leloir et al.[9]). A caveat here is that the tissue homogenate must be appropriately diluted prior to use. The dilution

is necessary to reduce the turbidity of the homogenate interference from competing enzymes/substrates in the homogenate. Furthermore, to account for NADH consumption that is not dependent upon glycogen synthase activity, blank reactions that contain all reaction components except UDP-glucose should be included. The glycogen synthase activity is then determined by subtracting the rate of change in NADH absorbance in the absence of UDP-glucose from that obtained in the presence of this compound.

Determination of glycogen phosphorylase activity

Like the glycogen synthase assay, the spectrophotometric measurement of phosphorylase activity can be performed in a continuous manner, while other phosphorylases assays are stopped assays[13]. It is also readily adaptable for use in microtiter plates or other high-throughput applications[15]. Again, its use with tissue homogenates requires appropriate dilution to reduce turbidity/interfering reactions. For example, glucose-6-phosphate is a rather abundant metabolite and its presence in a tissue homogenate will result in NADPH production *via* the glucose-6-phosphate dehydrogenase coupling enzyme independent of phosphorylase activity. When working with tissue homogenates, Control reactions that contain appropriately diluted tissue homogenate and all other assay components except phosphate and glycogen should be included. Phosphorylase activity is then calculated by subtracting the NADPH production in the absence of glycogen/phosphate from that which occurs in the presence of these compounds. Specific recommendations relating to the appropriate degree of dilution of various types of mammalian tissue extract can be found in Mezl et al.[13].

Determination of glycogen debranching enzyme activity

Although described here as a stopped assay, this procedure can be readily adapted and performed in a continuous fashion[16]. Since the assay relies upon the production of glucose, its use in crude tissue extracts must consider the release of glucose from phosphorylase limit dextrin by enzymes other than debranching enzyme, and the generation of glucose by the action of such enzymes on endogenous glycogen present in the tissue extract. The question of endogenous glycogen is easily addressed with a control reaction to which no phosphorylase limit dextrin is added. The presence of other α-glucosidase activities can be estimated in parallel reactions where maltose, rather than phosphorylase limit dextrin, is present as a substrate.

Determination of glycogen branching enzyme activity

Of the various quantitative assays discussed, the colorimetric branching enzyme assay is by far the least sensitive. Indeed, it has been estimated that the sensitivity is around 50–100 fold less than that achieved with the radiochemical method, where stimulation of the glycogen phosphorylase reaction by the addition of branching enzyme is measured[21]. Similar to the debranching enzyme assay, the branching enzyme assay is also sensitive to the presence of contaminating glucosidase activities, since digestion of the amylose will impact iodine binding. Some workarounds have been proposed to allow the use of assays similar to that described here in the presence of contaminating glucosidase activities[30]. However, in our view this assay is best suited to the study of purified or partially purified glycogen branching enzymes, where such interfering activities are minimal or absent.

Qualitative assessment of the extent of glycogen branching

The detailed analysis of glycogen branching is rather laborious, typically involving a combination of enzymatic digestion, chemical modification, and a variety of separation techniques to analyze the products generated[31]. While the colorimetric assay described here clearly cannot yield comparable information on the fine structure of glycogen, it does provide a simple and rapid measure of more or less branching. Furthermore, the spectra obtained do contain some additional information. For example, as discussed under Representative Results, samples of glycogen are typically present with absorbance peaks at ~400 nm and ~460 nm. The peak at ~400 nm apparently represents short outer chains in glycogen particles, since it is enhanced in glycogen isolated from yeast mutants lacking branching enzyme relative to wild type yeast[32].

ACKNOWLEDGMENTS:

The author would like to thank Karoline Dittmer and Andrew Brittingham for their insights and many helpful discussions. This work was supported in part by grants from the Iowa Osteopathic Education and Research Fund (IOER 03-17-05 and 03-20-04).

DISCLOSURES:

There are no known conflicts of interest associated with this work and there has been no financial support for this work that could have influenced its outcome.

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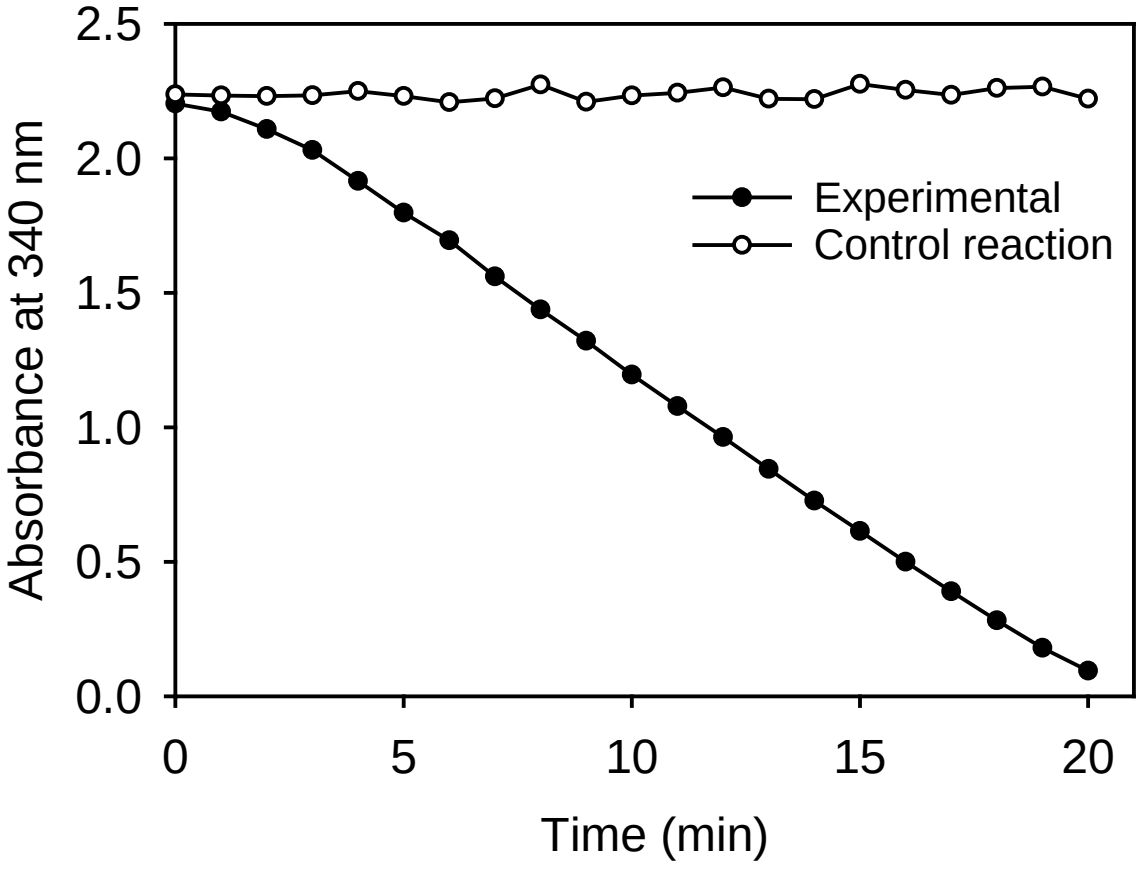
A.



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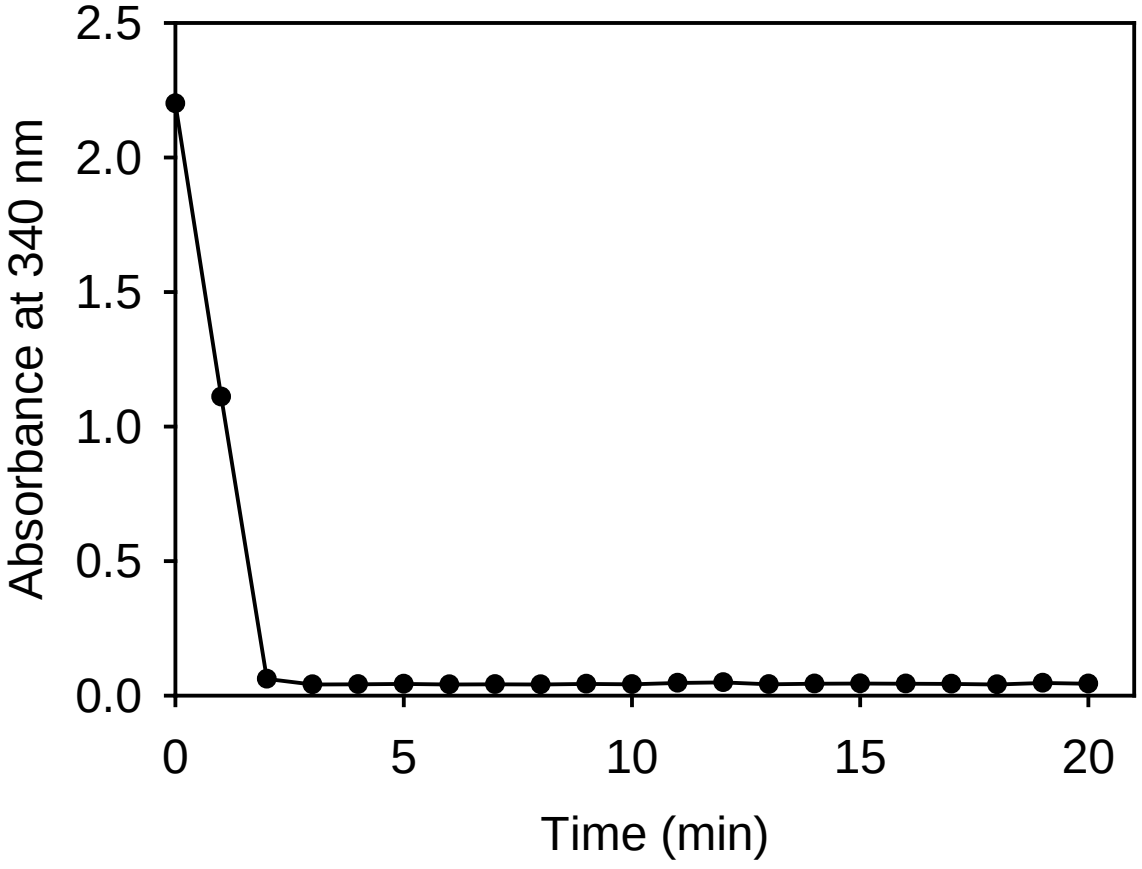

Figure 2

Click here to access/download;Figure;Figure 2.pdf

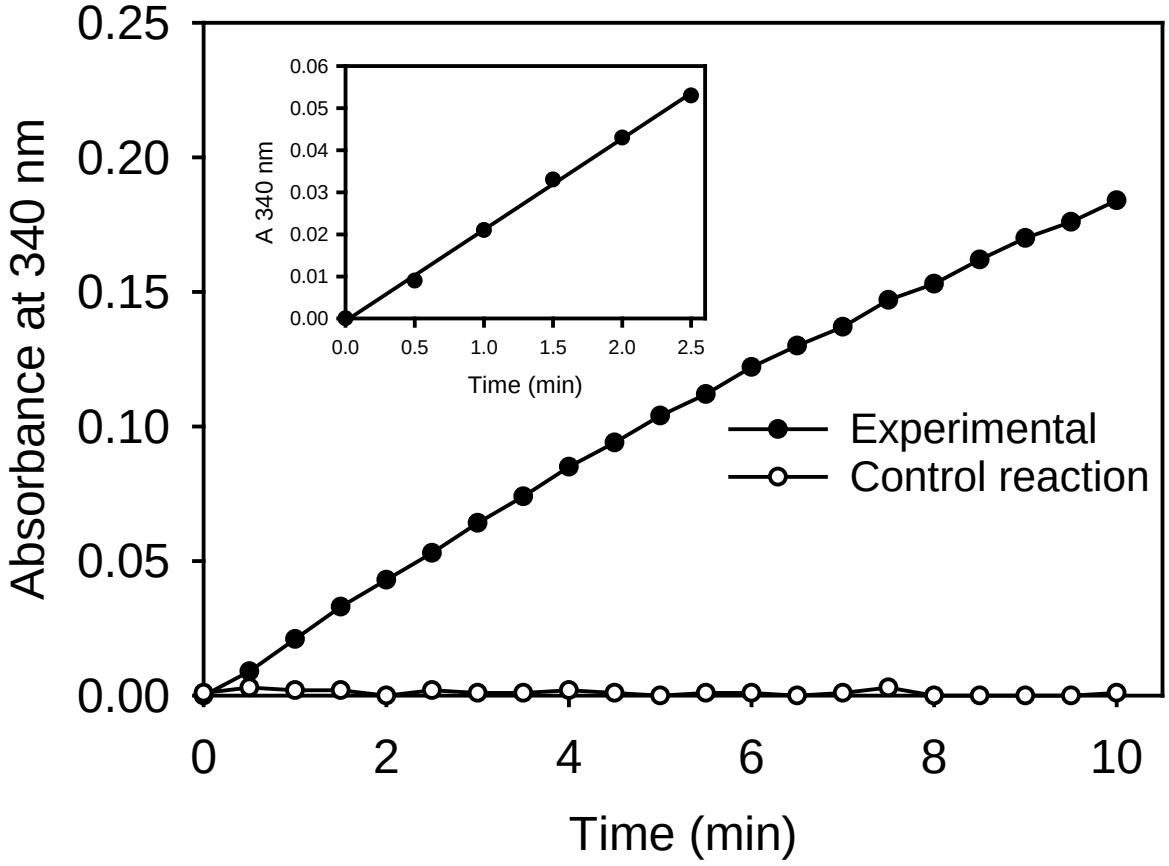

Figure 3

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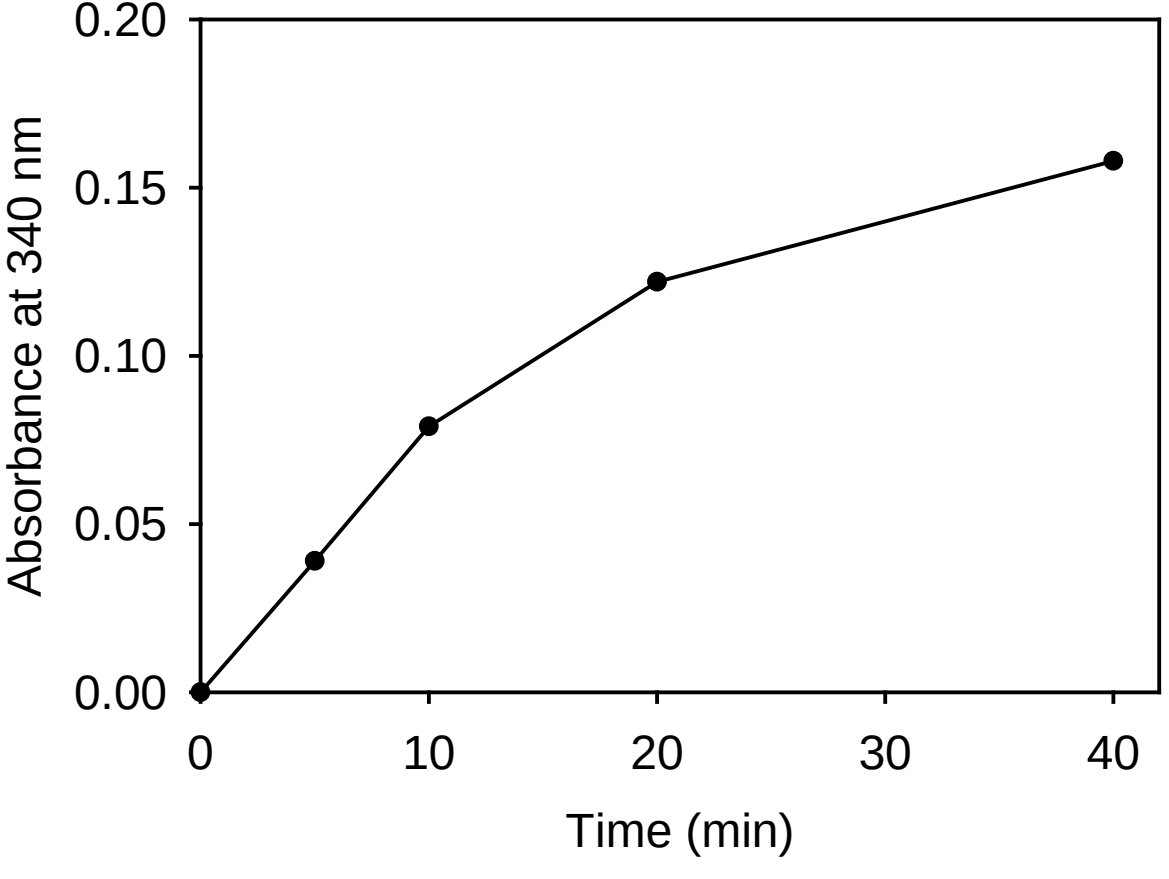
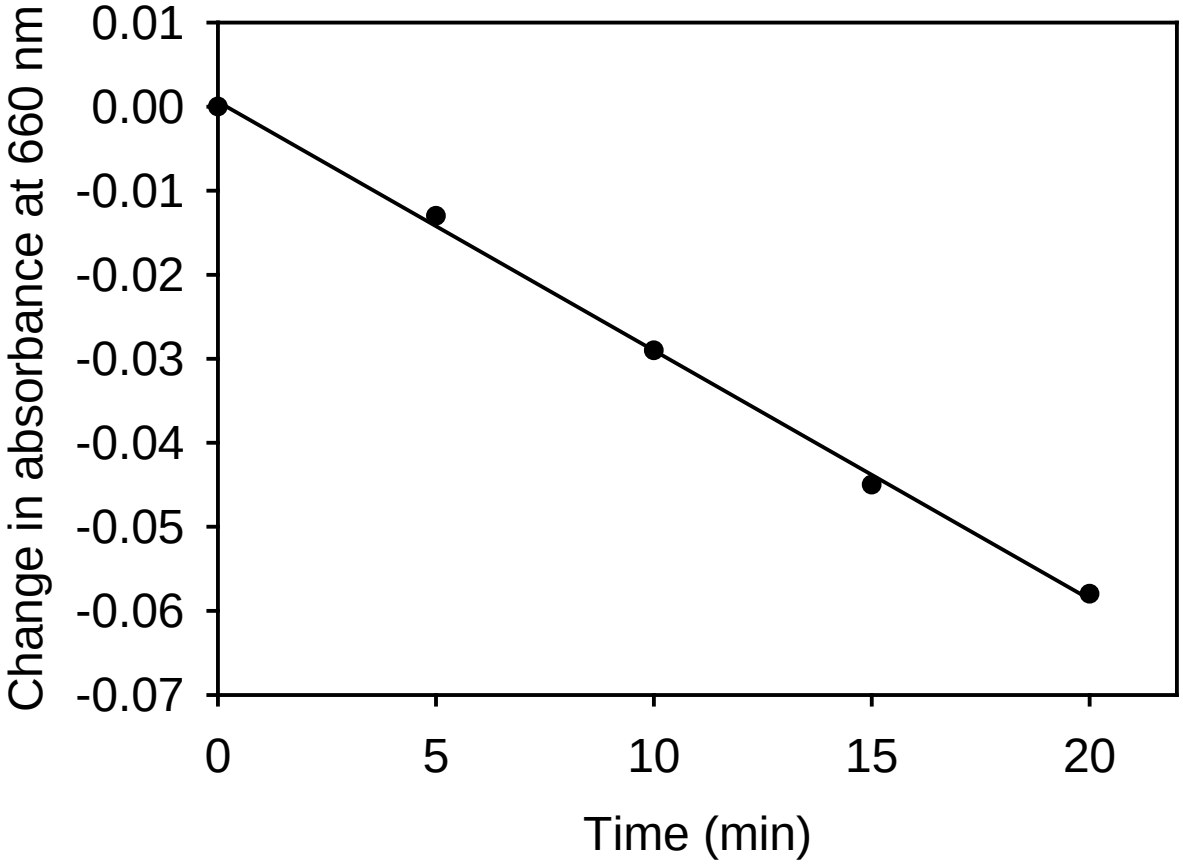


Figure 4

A.



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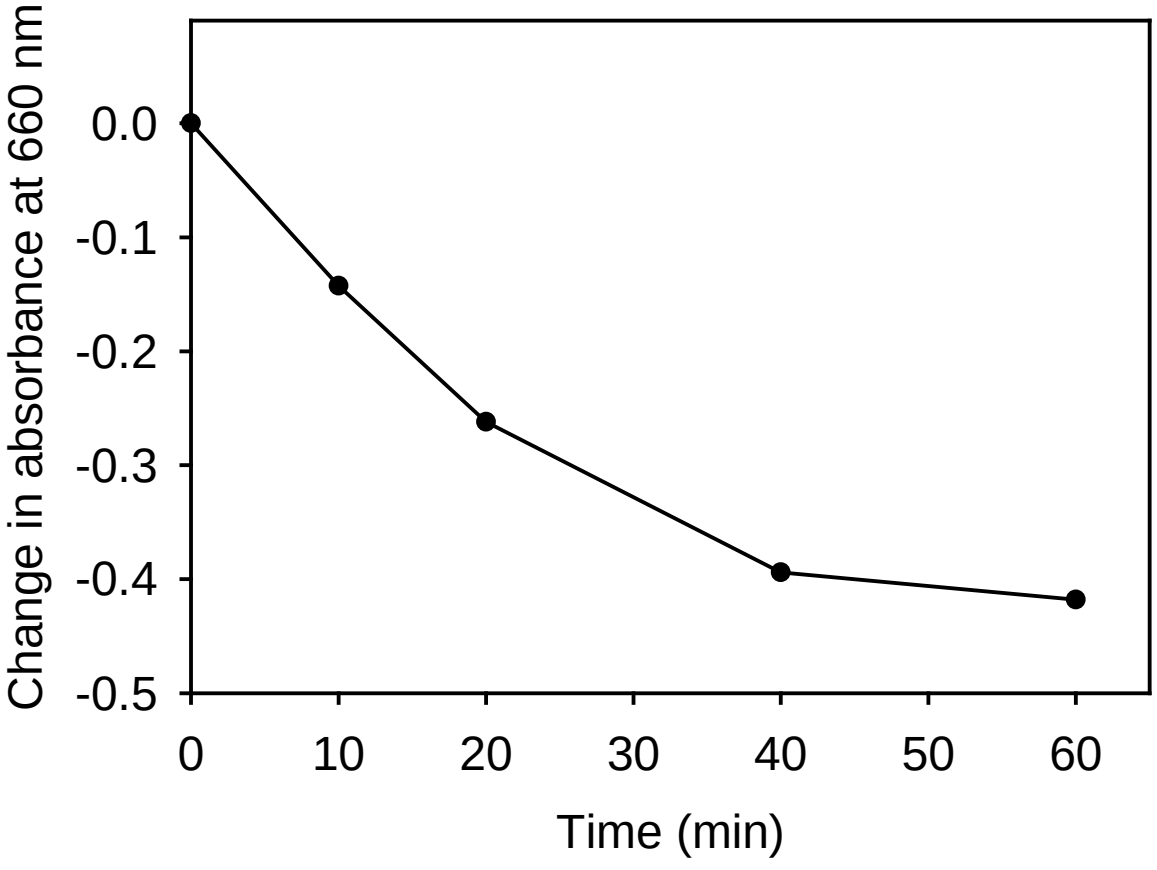
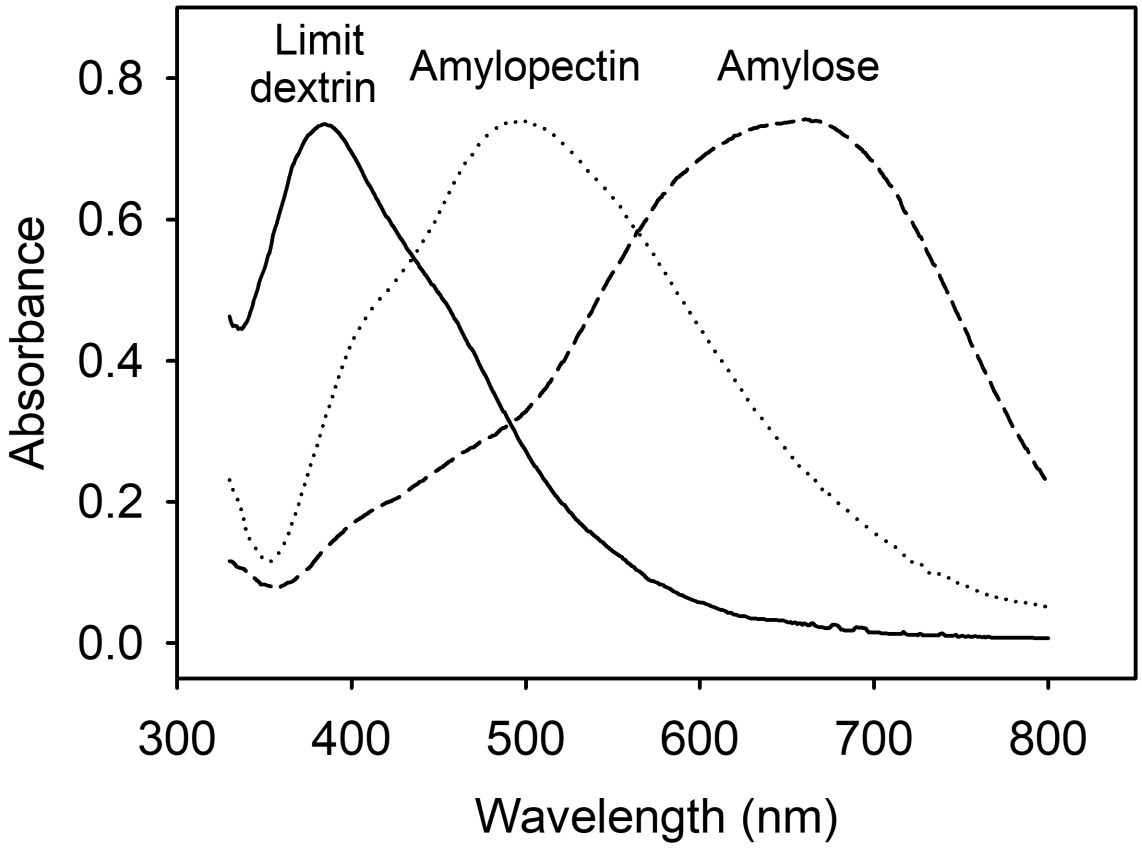


Figure 5

A.



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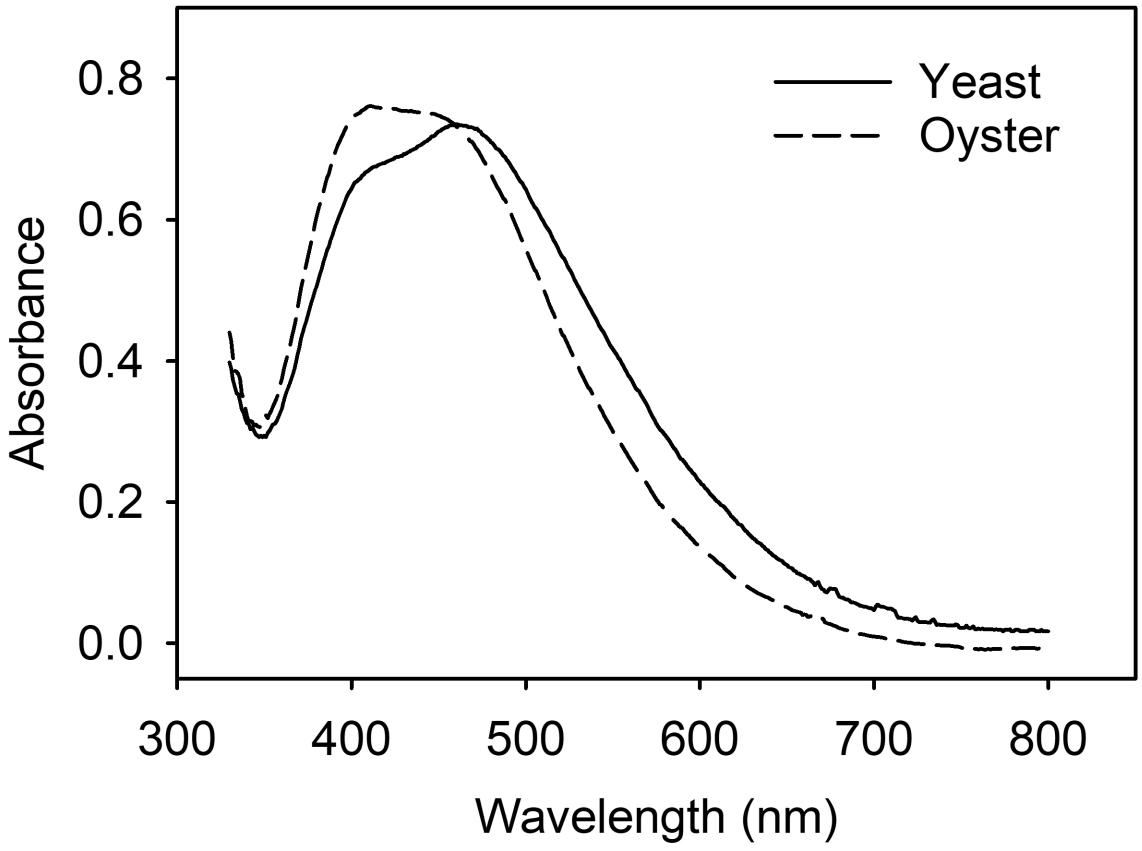

Component	Directions
50 mM Tris pH 8.0	Dissolve 0.61 g of Tris base in ~ 80 mL of water. Chill to 4 °C. Adjust the pH to 8.0 with HCl and make the volume up to 100 mL with water.
20 mM HEPES buffer	Dissolve 0.477 g of HEPES in ~ 80 mL of water. Adjust the pH to 7.0 with NaOH and make up the volume to 100 mL with water.
132 mM Tris/32 mM KCl buffer pH 7.8	Dissolve 1.94 g of Tris base and 0.239 g of KCl in ~90 mL of water. Adjust pH to 7.8 with HCl and make up the volume to 100 mL with water.
0.8% w/v oyster glycogen	Weigh out 80 mg of oyster glycogen and add to water. Make the final volume up to 10 mL with water and warm gently/mix to fully dissolve glycogen.
100 mM UDP-glucose	Dissolve 0.31 g of UDP-glucose in water and make the final volume up to 1 mL. Store in aliquots, frozen at -20 °C. Stable for several months.
50 mM ATP	Dissolve 0.414 g of ATP in ~ 13 mL of water. Adjust the pH to 7.5 with NaOH and make up the volume to 15 mL with water. Store in aliquots frozen at -20 °C. Stable for several months.
100 mM glucose-6-phosphate pH 7.8	Dissolve 0.282 g of glucose-6-phosphate in ~ 7 to 8 mL of water. Adjust the pH to 7.8 with NaOH. Make the volume up to 10 mL with water. Store frozen in aliquots at -20 °C. Stable for at least six months.
40 mM phosphoenolpyruvate	Dissolve 4 mg of phosphoenolpyruvate in 0.5 mL of 20 mM HEPES buffer pH 7.0. Store at -20 °C. Stable for at least 1 week.
0.5 M $MnCl_2$	Dissolve 9.90 g of $MnCl_2$ in a final volume of 100 mL water.
NDP kinase	Reconstitute lyophilized powder with sufficient water to give 1 U/µl solution. Prepare aliquots, freeze in liquid nitrogen, and store at -80 °C. Stable for at least 1 year.

Table 2

Component	Volume (µl)
160 mM Tris/32 mM KCl buffer pH 7.8	250
Water	179
100 mM glucose-6-phosphate, pH 7.8	58
0.8 % w/v oyster glycogen	67
50 mM ATP	80
4 mM NADH	80
100 mM UDP-glucose	28
40 mM phosphoenolpyruvate	20
0.5 M $MnCl_2$	8
Final volume	770

Component	Directions
125 mM PIPES pH 6.8	Dissolve 3.78 g of PIPES in water. Adjust the pH to 6.8 with NaOH and make up the volume to 100 mL with water.
8% w/v oyster glycogen	Weigh out 0.8 g of oyster glycogen and add to water. Make the final volume up to 10 mL with water and warm gently/mix to dissolve glycogen. Store frozen at -20 °C.
200 mM Na phosphate pH 6.8	Dissolve 2.63 g of $Na_2HPO_4.7H_2O$ and 1.41 g of $NaH_2PO_4.H_2O$ in water. Bring the volume up to 100 mL with water.
1 mM glucose-1,6-bisphosphate	Dissolve 2 mg of glucose-1,6-bisphosphate in 4 mL of water. Aliquot and store frozen at -20 °C. Stable for at least several months.
10 mM NADP	Dissolve 23 mg of NADP in 3 mL of water. Aliquot and store frozen at -20 °C. Stable for at least several months.

Table 4

Component	Volume (µl)
125 mM PIPES buffer pH 6.8	160
Water	70
8% w/v oyster glycogen	100
200 mM Na phosphate 6.8	400
1 mM glucose-1,6-bisphosphate	20
10 mM NADP	20
Final volume	770

Component	Directions
100 mM maleate buffer	Dissolve 1.61 g of maleic acid in ~ 80 mL of water. Adjust the pH to 6.6 with NaOH and make the final volume up to 100 mL with water.
300 mM triethanolamine hydrochloride/ 3 mM $MgSO_4$ pH 7.5	Dissolve 27.85 g of triethanolamine hydrochloride and 0.370 g of $MgSO_4.7H_2O$ in ~ 400 mL of water. Adjust the pH to 7.5 with NaOH and make up to a final volume of 500 mL with water.
150 mM ATP/12 mM NADP	Dissolve 1.24 g of ATP in ~ 10 mL of water. Monitor the pH and add NaOH to maintain a pH of ~ 7.5 as the ATP dissolves. Add 0.138 g of NADP. Adjust the pH to ~ 7.5 with NaOH and make up to a final volume of 15 mL with water. Store in aliquots at – 20 °C. Stable for several months.
50 mM Na phosphate buffer pH 6.8	Dissolve 32.81 g of $Na_2HPO_4.7H_2O$ and 17.61 g of $NaH_2PO_4.H_2O$ in water. Bring the volume up to a final volume of 5 L with water.

Response to Editorial and Reviewer Comments

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript…
I have proofread the manuscript and did not find any grammatical or spelling errors. I did, however, note that the phosphorylase assay procedure (line 199 onwards in the revised manuscript) had been partially truncated when I was adjusting formatting immediately prior to initial submission. Steps 2.5 through 2.9 have been added back. Only one non-standard abbreviation is used, NDP kinase, which is defined at first use. The other abbreviations used, such as ATP, NAD etc., are considered 'standard' per IUPAC-IUB and are typically used without further definition.

2. Please make sure the word count of the abstract is 150-300 words.
The abstract has been modified to address point 3, below, and is 160 words long.

3. Please revise the following lines to avoid overlap with previously published work…
Lines 14-15: Addressed
Lines 103-105: Addressed, see lines 110-113 in the revised manuscript
Lines 226-227, 233: I have not changed the language here. The technique of dialysis is being described. Essentially the same language will inevitably appear in any discussion of dialysis: material is dialyzed for a time in a dialysis bag, then transferred to another receptacle for further processing.
589-590: Addressed, see lines 630-633 in the revised manuscript.

4. Please ensure that all text in the protocol section is written in the imperative tense...
Addressed throughout. Text that could not be worded in the imperative has been moved into 'Notes'. Additional 'caution' statements have been added for use of caustic agents.

5. The Protocol should contain only action items that direct the reader to do something...
Addressed throughout the text.

6. Please note that your protocol will be used to generate the script for the video…
I believe that there is sufficient detail given in the protocol to allow easy replication.

7. In case you would like to include a few key steps for measurement of released glucose...
Agreed. Some steps in glucose measurement would be good to show and have been highlighted accordingly.

8. Please sort the Materials Table alphabetically by the name of the material.
Addressed. The table now in alphabetical order.

Reviewer's comments

I thank the reviewers for their careful appraisal of the manuscript and the thoughtful suggestions for revision that they have provided. Below, I address each point raised and detail how the manuscript has been modified in response.

Reviewer 1

The reviewer pointed out that the methods discussed focus on the enzymes of eukaryotic glycogen metabolism, rather than glycogen metabolism in a more general sense. I agree that this is the case and support changing the title of the manuscript to 'Spectrophotometric methods for the study of eukaryotic glycogen metabolism'.

The reviewer asked if there were any issues with employing crude extract or semi-purified enzymes in the assays described. Actually, most of the assays discussed were originally developed for use with crude extracts or partially purified enzymes. I have added language to clarify this point (lines 671-672). Language relating to the need to appropriately dilute cell extracts, and the requirement for additional control reactions when such samples are used, is already present in the manuscript (lines 673-679, 684-692, 698-704,714-716).

I indicated that allosteric effectors could be added to the buffers, should the enzyme under study require them (e.g. glucose-6-phosphate, in the case of glycogen synthase). The reviewer questioned if such effectors might impact the activity of the auxiliary enzymes used in the assay. This is an excellent point, which one must be cognizant of whenever a coupled enzyme assay is employed. Using the coupling enzymes described in the manuscript, purchased from the sources specified, glucose-6-phosphate at the concentrations typically employed does not impact coupling enzyme activity. Similarly, AMP can be added into the phosphorylase assay, at the concentrations typically used, without impacting coupling enzyme activity. If other effectors are to be investigated, then suitable control experiments will be required to ensure that it is the enzyme under study, and not one of the auxiliary enzymes, that is impacted. A clarifying note to this effect has been added to the text (lines 545-547).

Regarding the source of glycogen, yes, there are differences in the rate at which different glycogen synthases and phosphorylases act upon glycogen from different sources. Work in my own laboratory uses oyster glycogen as a 'standard' since the enzymes that we study show good activity towards this substrate and it is relatively inexpensive. As the reviewer suggested, I have added notes to the text to clarify the need to investigate different forms of glycogen (lines 500-504, lines 555-557).

The modification of the assays for use in a plate reader format has both advantages and disadvantages. We have used a plate reader ourselves for enzymatic glucose determinations very successfully, allowing rapid and highly parallel sample processing. Other workers, such as Goselin et al. (Anal. Biochem. 220:92-97, reference 26 in the manuscript) have described 96-well plate assays for certain enzymes of glycogen metabolism. Our experiences with the use of

plate readers for kinetic analysis have been rather mixed, perhaps reflective of the instrumentation we were using, much reduced sample volumes, or even plate quality. For the current manuscript, I chose to focus on the simplest possible approaches that still yield high quality data.

Reviewer 2

1. The reviewer suggested inclusion of a brief sentence in the introduction about the relevance/importance of the study of glycogen metabolism. Such a statement has been added (lines 30 to 35).
2. The reviewer suggested adding an image, showing the different colors of glycogens with different degrees of branching. Given the visual nature of the Journal, I believe this is an excellent suggestion. However, while it is easy to see the difference between amylopectin/iodine and glycogen/iodine complexes by eye, a spectrophotometer is needed to detect the more subtle difference between, for example, oyster glycogen/iodine and rabbit liver glycogen/iodine complexes. Therefore, I do not believe that it would be possible to capture an image that was helpful to a reader or viewer.