Chapter 2

Extraction, Assay, and Light Activation of NADP+-3-Phosphoglyceraldehyde Dehydrogenase

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Contents

Introduction

Photosynthesis in higher plants can be summarized as:

$$2nH_2O + nCO_2 \xrightarrow{\text{light}} (CH_2O)n + nO_2 + nH_2O$$

Conveniently, it can be divided into two distinct processes: (a) conversion of light energy into chemical energy (photosynthetic electron transport) and (b) CO2 reduction (the Calvin-Benson-Bassham cycle). The pigment systems of the thylakoid phase of the chloroplast first absorb light energy, which results in phosphorylation of ADP and reduction of NADP+. (A list of abbreviations appears in Appendix A.) Then, in the so-called "dark reactions" of the chloroplast, the formed ATP and NADPH are used to reduce CO2 to carbohydrate. Actually, the relationship between these processes extends to subtle regulatory aspects. The purpose of this exercise is to demonstrate that the extracted activity of one of these enzymes depends on the physiological state of the plant. In addition, the instructor may, as we have done at Florida State, use this exercise as an introduction to an open-ended project. Our experience indicates that such an approach is more challenging and stimulating than a prescribed set of mechanical cookbook exercises. Finally, depending on the time available and the level of the students, the instructor can use the appendices to teach students how to make pH-buffered solutions and to keep a laboratory notebook.

The metabolite interconversions of the Calvin-Benson-Bassham cycle are detailed in most basic biology books. Here, we will give only an overview. RuBP carboxylase catalyzes the carboxylation of the 5-C acceptor molecule RuBP to yield two molecules of the 3-C phosphorylated acid PGA. PGA is phosphorylated to 1,3-diPGA (an ATP is used for each of the two PGAs). The reductive step of the Calvin-Benson-Bassham cycle is the conversion of 1,3-diPGA to PGAL (an NADPH is used for each of the two 1,3-diPGAs):

$$1,3\text{diPGA} + \text{NADPH} \longrightarrow \text{PGAL} + \text{NADP+} + \text{Pi} \quad (1)$$

which is catalyzed by PGAL dehydrogenase (DH). In brief, the carbons of PGAL are subsequently rearranged, in several steps, to Ru5P. Ru5P is phosphorylated to yield the acceptor molecule RuBP (an ATP is used). Most of the metabolite interconversions of the Calvin-Benson-Bassham cycle are shared by other pathways, such as the oxidative pentose phosphate pathway and glycolysis. For example, the reaction shown in equation (1), above, is the "reverse" of the oxidative step of glycolysis, except for pyridine nucleotide specificity. As a result, mechanisms have evolved that prevent "futile cycling" of carbon and the attendant energy loss. Specifically,

all three unique enzymes in the Calvin-Benson-Bassham cycle (i.e., RuBP carboxylase, PGAL DH, and Ru5P kinase) as well as the phosphatases are more active in a plant in light than in one in darkness. Conversely, flux through the oxidative pentose phosphate pathway is diminished by light-mediated enzyme deactivation. The exact mechanisms are not the same for all the enzymes. For example, RuBP carboxylase is thought to be activated by light-mediated changes in the stromal proton and magnesium concentrations and by a specific enzyme activase that may be involved in carboxylating the enzyme, whereas phosphatase activation probably results from light-mediated disulfide reduction of the enzyme. Nevertheless, one principle applicable to all these cases has emerged: light has no direct effect on the enzymes themselves; activation is ultimately a result of photosynthetic electron transport. Thus, in this general way, the activities of the two "distinct" processes of photosynthesis, as outlined in the introductory paragraph, are coordinated.

From the preceding overview, it follows that light-regulation of enzymes can be placed in one of two broad categories. The first is modification of the chemical environment, for example, as exemplified by part of the activation of RuBP carboxylase. In vitro "activation" depends upon simulation of the presumed in vivo milieu. The second type of regulation involves reversible modification of the regulated enzyme itself. These enzymes can be extracted in a more active or a less active form and therefore lend themselves easily to exercises, which allow the students to correlate extractable catalytic rate with the physiological condition of the plant. In this exercise, one enzyme, PGAL DH (equation 1) will be studied. PGAL DH, though not activated to the extent that several other enzymes are, was the first activity found to be light activated and is simple to assay. For general reviews, the instructor is directed to Anderson et al. (1976, 1980, 1982), Bergmeyer (1978), Buchanan (1980), Buchanan et al. (1979), Huber (1978), Lendzian and Zeigler (1978), Wirtz et al. (1982), and Ziegler et al. (1969).

The students will monitor the reaction in equation (1) spectrophotometrically in real time. The reaction indicator is the oxidation of the substrate, NADPH. The peak absorbance of NADPH is at approximately 340 nm. NADP+, the oxidized form that is produced, has negligible absorbance between 300 and 380 nm. Thus, the student will record decline in A340 as a function of time.

The initial exercise is designed to take 3 hours, but it can serve as an introduction to an open-ended semester-long research project suitable for third-year students and also includes appendices on preparation of buffered solutions and preparation of a laboratory notebook. Preparation of stock solutions (Appendices B and C) should take approximately 6 hours. Instructions are given in Appendix D for preparation of plant materials; species can be raised from seed that take 1, 2, or 3 weeks to mature, or spinach leaves can be purchased at a supermarket.

Materials

Stock Solutions (Quantities are specified in Appendices B and C)

2 M Tris(base)
1 M HCl
50 mM MgCl2
5 mM EDTA

Equipment

Razor blade (for tissue harvest)
100-ml graduated cylinder (for cocktail preparation)
Ten-Brock or other tissue homogenizer
Refrigerated centrifuge and 15-ml tubes
Spectrophotometer and cuvets (four per work station)
pH meter
Micropipets
Petri dishes
"Dark box" for incubation of leaf tissue

Other

Ice for chilling
Plant material

Notes for the Instructor

Because it is expensive, the other substrate, 1,3-diPGA, will not be added directly to the cocktail. Instead, it will be synthesized there. (For this reason, the cocktail contains ATP, Mg^{2+}, and PGAK; when PGA is added, it is converted to 1,3-diPGA.) Conversion of PGA to 1,3-diPGA takes 2–5 minutes, so the apparent reaction rate will not be linear immediately upon addition of PGA.

If a spectrophotometer is not available, a photometer with isolatable Hg334 or Hg365 lines can be used as a substitute.

Student Outline

Experimental Summary

The purpose of this exercise is to demonstrate that the activity of enzymes controlling the reactions of the Calvin-Benson-Bassham cycle depends on the physiological state of the plant. This dependence is an adaptive response that prevents "futile cycling" and the attendant energy loss; modification of enzyme activity by exposure to light or darkness ensures that the "light reactions" of photosynthesis will be coordinated with the "dark reactions." The enzyme to be studied here is NADP+-3-phosphoglyceraldehyde dehydrogenase (PGAL DH, EC 1.2.1.13), the activity of which is affected by exposure of the leaf to light.

The following order of experimentation is recommended: (1) preparation of crude enzyme extract from leaves; (2) assay evaluation; (3) extraction and assay of activity from leaves incubated in light or in darkness; and (4) open-ended exercises, for example, determination of the time course for activation and the kinetic constants, inhibition of photosynthetic electron transport (to block activation), substitution of reducing agents for the light requirement, determination of the action spectrum for activation, and other experiments devised by the student.

A list of abbreviations appears in Appendix A.

Procedures

Tissue Pretreatment

1. Cut a piece weighing 30–100 mg (approximately 4 cm2) from a leaf blade.
2. Weigh the leaf piece and float it on water in a petri dish.
3. Place the dish in darkness for minimum activity or under illumination for maximum activity.
4. Incubate it for 15 minutes.

Cocktail Preparation

1. Prepare 25 ml extraction cocktail containing 100 mM Tris-Cl (pH 7.8), 1 mM MgCl2, and 100 μM EDTA using stock reagents at the work station. Store it on ice. (See Appendix B.)
2. Prepare 60 ml assay cocktail containing 100 mM Tris-Cl (pH 8.1), 10 mM MgCl2, 1 mM ATP, 150 μM NADPH, 0.5 μg/ml PGAK, and 0.02% (w/v) BSA from stock reagents at your work station. Store the cocktail at room temperature. (See Appendix C.)

Tissue Extraction

1. Homogenize the pretreated leaf piece well in 1 ml of precooled (about 4°C) extraction cocktail. It is important to minimize time between the end of pretreatment and tissue disruption (to prevent inadvertent changes in activation state).
2. Centrifuge (at least 500 × g) the extract for 5 minutes.
3. Remove the clear, cell-free extract and retain it on ice for subsequent assay. Discard the particulate pellet. If necessary, repeat the centrifugation. Store the extract on ice.

Assay Validation

Before actual studies on the light activation of PGAL DH are undertaken, the assay must be validated. There are several ways this validation can be accomplished, but for an established assay, it is usually sufficient to show that the reaction indicator (in this case, diminution of A340) is substrate-dependent, linear with time, and linear with extract amount. To this end:

1. Set up four spectrophotometer tubes, each containing 3 ml of assay cocktail. Amend the first two tubes to contain 2 mM PGA. Initiate the reaction by adding 25 μl of extract to Tubes 1 and 3. The result should be:

   Tube 1: + PGA, + extract
   Tube 2: + PGA, - extract
   Tube 3: - PGA, + extract
   Tube 4: - PGA, - extract

2. Using water to "blank" the spectrophotometer, determine the A340s of the four tubes, in order, and record the time of each reading. Repeat the readings, still in order, until one tube has declined by approximately 0.4 optical density (O.D.).

3. Plot the A340 of each tube against time. Calculate the slopes ($\Delta A340/\Delta time$).

4. Before proceeding, analyze your data: (a) Was the reaction rate in Tube 1 substantially faster than that in the controls, Tubes 2 to 4? (b) Was the reaction rate too fast to monitor accurately (or too slow for convenience)? For subsequent experiments, alter the aliquot volume accordingly. (c) Was the reaction rate linear with time? (d) Was substrate-independent NADPH oxidation significant? (e) Calculate the specific activity (see Appendix D).

5. Devise other experiments to validate the assay, as time permits: (a) Repeat the above experiment after the extract has aged, to verify that the activity and reagents are stable. (b) Assay several extract aliquot volumes, to determine whether the activity is proportional to extract volume (as it should be). (c) Attempt to denature the enzyme (e.g., by heating an extract aliquot) before assay.

Experiments on PGAL DH Activity

After you are convinced your assay for PGAL DH is valid, devise experiments to describe the phenomenon of light activation:

(a) Prepare an extract from a leaf incubated in darkness and one from a leaf incubated in the light. Compare their specific activities. To what extent is PGAL DH activated by light? Does concomitant assay of a mixture of the two extracts indicate a dissociable inhibitor in the extract or populations of "activated" and "inactivated" enzyme?

(b) Prepare extracts from leaf pieces incubated in light for various lengths of time. What is the time course for activation?

(c) Prepare extracts from leaf pieces incubated in the dark for various lengths of time. What is the time course for deactivation?

(d) Prepare extracts from other plant organs (e.g., stem or root). How does the specific activity there compare to that of leaf? Is this activity affected by exposure to light?

Suggestions for Further Exercises

After the phenomenon of light activation has been described, devise experiments to elucidate the mechanism:

(a) How much light is required for activation? Expose the leaf pieces with dim or strong light before extraction and assay.

(b) Is photosynthetic electron transport necessary for activation? Under a solution of 50 μM DCMU (which inhibits linear electron transport), sever the petiole of a plant incubated in darkness. Incubate the severed leaf in darkness for 60 minutes (to "pull" the DCMU solution into the leaf, through the transpiration stream). Illuminate this leaf and a control leaf for 15 minutes. Extract and assay portions of these leaves.

(c) Can a reductant substitute for light in the activation of PGAL DH? Amend an extract from a leaf incubated in the dark to include 100 mM dithiothreitol. Incubate for 1 hour. Assay and compare to control.

Typical Student Data (for Extracts of Pea Leaf)

Decline in O.D./minute (50 mg of illuminated leaf extracted in 1 ml extraction cocktail, 40 µl extract):

(a) complete reagent: 0.040
(b) complete reagent – extract: < 0.001
(c) complete reagent – PGA: < 0.002

Specific activity (illuminated leaf):

9 mmol·min-1·kg-1

Linearity of reaction with time (4–12 minutes):

$R^2 = 0.99$, n = 5 (time points)

Linearity of reaction with extract aliquot size (10–40 ml):

$R^2 = 0.96$, n = 4 (aliquot volumes)

Reproducibility of specific activity (illuminated leaf):

$SD/\bar{x} = 0.15$, n = 3 (extracts from different leaves)

Reproducibility of specific activity (leaf incubated in darkness):

$SD/\bar{x} = 0.08$, n = 3 (extracts from different leaves)

Time course for light activation (400 µE·m-2·s-1):

$t_{½} \cong 2.5$ minutes

Time course for dark inactivation:

$t_{½} \cong 4$ to 10 minutes

Extent of light activation (percent of dark control, 30 minutes light):

µE·m-2·s-1	Percentage
200	220
250	200
300	165
400	314
700	300

DCMU-inhibition of light activation (percent of light control):

42% (i.e., similar to dark control)

Non-physiological activation (+ 100 mM dithiothreitol, 1 hour, 23°C, percent of control [= different aliquot of same extract from dark leaf, incubated similarly, but without dithiothreitol]): 558%

Literature Cited

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APPENDIX A
Abbreviations

ATP:	adenosine-5′-triphosphate
BSA:	bovine serum albumin
EDTA:	ethylenediamine tetraacetate
NADP:	nicotinamide adenine dinucleotide phosphate
PGA:	3-phosphoglycerate
PGAK:	3-phosphoglycerate kinase (EC 2.7.2.3)
PGAL:	3-phosphoglyceraldehyde
PGAL DH:	NADP+-3-phosphoglyceraldehyde dehydrogenase (EC 1.2.1.13)
Ru5P:	ribulose-5-phosphate
RuBP:	ribulose-1,5-bisphosphate

APPENDIX B
Preparation of Extraction Cocktail

The following stock solutions are required for each student or group of students working together: (1) 12 ml 2 M Tris(base); (2) 12 ml 1 M HCl; (3) 40 ml 50 mM MgCl2; and (4) 1 ml 5 mM EDTA. The quantities given below and in Appendix C are sufficient for the preparation of one extraction cocktail and three assay cocktails at each of 25 work stations.

1. 2 M Tris(base): Dissolve 72.6 g Tris(base) in H2O, qs to 300 ml. Stable; for storage, maintain at -20°C.
2. 1 M HCl: Dilute 25 ml 12 N HCl (sp. gr. 1.19, 38% w/w) to 300 ml with H2O. Stable; for storage, maintain at -20°C.
3. 50 mM MgCl2: Dissolve 10.1 g MgCl2·6H2O in H2O, qs to 1 liter. Stable; for storage, maintain at -20°C.
4. 5 mM EDTA: Dissolve 0.2 g Na2·EDTA in H2O, qs to 100 ml. Stable; for storage, maintain at -20°C.

To prepare 25 ml extraction cocktail (i.e., sufficient for 20 extractions), the students will calculate the formulation:

1. To a 25 ml mixing cylinder, add 1.25 ml 2 M Tris(base), 1.7 ml 1 M HCl, 0.5 ml 50 mM MgCl2, 0.5 ml 5 mM EDTA, and H2O to make 25 ml, mixing after each addition.
2. Remove 5 ml for pH verification by electrode. The extraction cocktail is stable; store on ice. The tissue extract is stable at 4°C for at least 2 hours.

   Note: Tris buffers against pH changes. Mg2+ provides stability of enzyme activity. EDTA provides chelation of divalent cations. Coolness minimizes protease activity, etc., in the extract.

APPENDIX C
Preparation of Assay Cocktail and Amendments

In addition to the stock solutions described in Appendix B, each student or group should be provided with (1) 2 ml 100 mM ATP, (2) 2 ml 50 µg/ml PGAK, (3) 2 ml 200 mM PGA, (4) 3 ml 10 mM NADPH, and (5) 2 ml 1% (w/v) BSA.

1. 100 mM ATP: First, prepare 50 ml 0.2 M NaOH (= 0.4 g NaOH dissolved in 50 ml H2O). This diluent is stable at room temperature. Dissolve 3 g Na2·ATP in diluent, qs to 50 ml. Stable at 4°C; for storage, maintain at -20°C.
2. 50 µg/ml PGAK: First, make 50 ml 20 mM Tris-Cl (pH 8.1) containing 0.02% (w/v) BSA (to 0.5 ml 2 M Tris(base) add 0.5 ml 1 M HCl, dilute to 40 ml with water, add 10 mg BSA, qs to 50 ml with H2O and mix). This enzyme diluent is stable at -20°C. Add 0.25 ml 10 mg/ml PGAK (commercial suspension) to 50 ml diluent. Stable at 4°C for 1 week.
3. 200 mM PGA: Dissolve 2.3 g Na2 PGA in H2O, qs to 50 ml. Stable at 4°C; for storage, maintain at -20°C.
4. 10 mM NADPH: First, prepare 50 ml 100 mM carbonate (pH ~ 10.0) (= 0.8 g Na2CO3 + 0.2 g NaHCO3 dissolved in 100 ml H2O). This diluent is stable at -20°C. Add 0.75 g Na4·NADPH to 75 ml diluent. Stable at 4°C for 1 week. (Alternatively, it can be stored at -50°C or colder. Do not store at -20°C.)
5. 1% BSA: Dissolve 500 mg BSA in H2O, qs to 50 ml. Stable at 4°C; for storage, maintain at -20°C.

To prepare 60 ml assay cocktail (i.e., sufficient for 18 assay tubes), the students will calculate the formulation:

1. To 30 ml H2O in a 100 ml mixing cylinder, add 3 ml 2 M Tris(base), 3 ml 1 M HCl, 12 ml 50 mM MgCl2, 0.6 ml 100 mM ATP, 0.6 ml 50 µg/ml PGAK, 0.90 ml 10 mM NADPH, 1.2 ml 1% (w/v) BSA, qs to 60 ml with H2O, mixing after each addition.
2. Remove 5 ml for pH verification by electrode. The assay cocktail is stable for at least 2 hours at 25°C. The substrate generator, PGA, will be added to each tube (containing 3 ml assay cocktail), as required, to a concentration of 2 mM (= + 30 µl 200 mM PGA). The reaction will be initiated by addition of extract (= + 10 to 75 µl).

Note 1: PGA, PGAK, ATP, and Mg2+ are to synthesize 1,3diPGA. Tris buffers against pH changes. NADPH is a substrate (and reaction indicator). BSA stabilizes PGAL DH activity.

Note 2: For students who wish to mimic light activation of PGAL DH, 0.5 g dithiothreitol (DTT), a reductant, can be made available.

Note 3: For students who wish to prevent light activation by blocking photosynthetic electron transport, 2 g 3-(3,4 dichlorophenyl)-1,1-dimethyl urea (DCMU, the herbicide diuron) can be made available.

APPENDIX D
Plant Materials

Light activation of PGAL DH is a general phenomenon that is found in many taxonomically diverse plants. We ourselves have experience with four sources:

1. Avena sativa (oats, cv. "Victory"): 3.2x stimulation by light (~ 400 μE·m-2·s-1, 1 experiment). Soak seeds in distilled water for 4 hours. Plant 2 cm deep in moist vermiculite. Place on window sill. Use 1-week-old seedlings.

2. Pisum sativum (English or garden pea, cv. "Progress No. 9"): See Typical Student Results section. Dust seeds with Captan. Plant 2 cm deep in moist vermiculite. Place on window sill. Use 2-week-old seedlings.

3. Vicia faba (broad bean, cv. "Longpod"): 1.6x stimulation by light (~ 300 μE·m-2·s-1, 1 experiment.) Plant seeds 3 cm deep in "Hall's All Purpose Potting Soil" (Largo, FL) amended with 5 g/liter 6-6-6 and 1 ml/liter 1 M MgSO4. Maintain light >500 μE·m-2·s-1 and 14 hours per day (22°C/18°C). Use 3-week-old plants.

4. Market spinach (leaf spinach purchased in a supermarket): 2.9x stimulation by light (~300 μE·m-2·s-1, 1 experiment).

APPENDIX E
Calculation of PGAL DH Specific Activity

For convenience, the specific activity calculations are made on a mass basis. A contrived example will be used to demonstrate how this is done.

Step 1: Calculation of fresh-mass-equivalent in extract

Suppose 50 mg of fresh leaf is homogenized in 1 ml extraction cocktail. Assume fresh leaflet is 85% water. Then, the total soluble phase of the extract is 1.000 ml + (0.85 × 0.05 g) = 1.042 ml. Thus, each microliter of the soluble extract phase would be equivalent to 48 × 10-9 kg fresh leaf; i.e., (50 × 10-6 kg)/1042 µl. Or, 30 µl extract is equivalent to 1.44 × 10-6 kg leaf.

Step 2: Calculation of substrate reacted per unit time

Assume:

	A340 (5 minutes)	A340 (15 minutes)	ΔA340/minute
Complete reagent	0.80	0.51	0.029
Complete reagent - PGA	0.82	0.77	0.005
Complete reagent - extract	0.74	0.74	0.000

Thus, the extract-dependent, substrate-dependent decline in A340 is 0.024 O.D./minute. The millimolar extinction coefficient of NADPH (1 cm light path) is 6.270 at 340 nm (which, at the accuracy of these experiments, can be considered independent of experimental variables). Thus, a solution of 0.16 mM (= 1/6.27) has an A340 of 1 O.D. It follows that a ΔA340/Δtime of 0.024 O.D./minute = 3.84 µmolar/minute [= (0.16 mM/O.D.) × (0.024 O.D./minute)].

In a total volume of 3.06 ml (= 3 ml cocktail + 0.03 ml extract + 0.03 ml PGA), the substrate consumed is 11.8 nmol/minute [= (3.06 × 10-3 liter) × (3.84 × 10-6 mol/liter)/minute].

Step 3: Calculation of specific activity

Specific activity is defined as substrate reacted·time-1·tissue-1. Thus, the specific activity = 11.8 nmol·minute-1·(1.44 × 10-6 kg)-1 ≈ 8.2 mmol·minute-1·kg-1.

APPENDIX F
Preparation of a Buff*E*rred Solution

Most students do not know how to prepare a pH-buffered solution properly. This appendix can be incorporated into the exercise at the instructor's discretion.

The Hendersen-Hasselbalch equation,

is used to calculate the ratio of the base concentration to the acid concentration for a desired pH. (Note: The absolute concentrations of the base and acid are unimportant, assuming a constant pK.)

$$pH = pK + \log\left(\frac{base}{acid}\right)$$

Example

Tris(base) is supplied as a 2 M "stock" solution. The desired "working" concentration of Tris is 100 mM at pH 7.8. Tris has a pK of 8.1. The pH will be adjusted with 1 M HCl. To prepare 25 ml of "extraction cocktail":

Step 1: Calculate and make the required dilution from the stock

$$\frac{2\ M\ \ (stock)}{100\ mM\ \ (working)} = \frac{2000\ mM}{100\ mM} = 20x\ dilution$$

Thus, to a 25-ml mixing cylinder, add 1.25 ml of 2 M stock Tris(base) (1.25 ml = 1/20 × 25 ml).

Step 2: Calculate the ratio of base to acid required, using the Hendersen-Hasselbalch equation

$$7.8 = 8.1 + \log\left(\frac{base}{acid}\right)$$

$$\log\left(\frac{base}{acid}\right) = -0.3$$

$$\log\left(\frac{acid}{base}\right) = 0.3$$

$$\frac{acid}{base} = 2$$

As the pH will be adjusted with HCl,

$$\frac{Tris - Cl}{Tris\ (base)} = 2$$

For a total Tris concentration of 100 mM,

$$\frac{[\ 2\ parts\ Tris\text{-}Cl\ ]}{[\ 2\ part\ Tris\text{-}Cl\ ] + [\ 1\ part\ Tris\ (base)\ ]}\ x\ 100\ mM = \frac{2}{3}\ x\ 100\ mM = 67\ Tris\text{-}Cl$$

Similarly,

$$\frac{[\ 1\ part\ Tris\ (base)\ ]}{[\ 2\ part\ Tris\text{-}Cl\ ] + [\ 1\ part\ Tris\ (base)\ ]}\ x\ 100\ mM = \frac{1}{3}\ x\ 100\ mM = 33\ Tris\ (base)$$

Step 3: Calculate and make the dilution required to achieve the desired Tris-Cl concentration

$$\frac{1\ M\ (stock\ H^{+})}{67\ mM\ (working)} = \frac{1000\ mM}{67\ mM} = 15x\ dilution$$

Step 4: To the mixing cylinder, add about 15 ml water and mix

Step 5: Add calculated aliquots of other stock reagents (MgCl2, EDTA)

Although mixing between additions of these particular reagents is not necessary, it is "good lab practice," as it can be important in other cocktails.

Step 6: Bring the final volume to 25 ml with water and mix

Step 7: Remove a small aliquot and check the pH by electrode

Typically, the pH estimate obtained on a pH meter for Tris buffer is 0.2 units lower than the actual pH.

Exercise

(a) Have students complete Table 2.1 (on the following page).

(b) Which is the weakest buffer/pH combination for buffering against acid addition or formation?

Answer: Buffer X, 50 mM, pH 7

(c) Which is the weakest buffer/pH combination for buffering against base addition or formation?

Answer: Buffer Y, 50 mM, pH 8.5.

(d) At pH 8, why would you use Buffer X at 50 mM instead of Buffer Y at 100 mM? To answer this question, consider the effect of adding or forming 5 mM acid or base in each solution.

Answer:
Buffer X + 5 mM acid = 30 mM acid: 20 mM base; pH = 7.8
Buffer X + 5 mM base = 20 mM acid: 30 mM base; pH = 8.2
Buffer Y + 5 mM acid = 29 mM acid: 71 mM base; pH = 7.6
Buffer Y + 5 mM base = 19 mM acid: 81 mM base; pH = 8.6
Thus, the pH change in Buffer X is less than the pH change in Buffer Y.

Table 2.1. Acid:base ratios for different buffers at a range of pHs.

pH	7.0		7.4		7.5		7.6		7.7		7.8		7.9		8.0		8.1		8.5	
Acid (A) or Base (B) in mM	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Buffer X, pK 8.0 100 mM	91	9	80	20	76	24	72	28	67	33	61	39	56	44	50	50	44	56	24	76
Buffer X, pK 8.0 50 mM	45	5	40	10	38	12	36	14	33	17	30	20	28	22	25	25	22	28	12	38
Buffer Y, pK 7.5 100 mM	76	24	56	44	50	50	44	56	39	61	33	67	28	72	24	76	20	80	9	91
Buffer Y, pK 7.5 50 mM	38	12	28	22	25	25	22	28	20	30	27	33	14	36	12	38	10	40	5	45

APPENDIX G
How to Keep a Laboratory Notebook

Maintenance of laboratory records is one of the most neglected aspects scientific training. Undergraduates are taught laboratory practice by repetition of short, discrete exercises, followed by externally generated reports, but are later expected, as graduate students or professional researchers, to join a system of research that ultimately depends on long-term records and planning. Although philosophies differ, it is the opinion of these authors that record books are property of the laboratory, where they form a "library" of experiments (just as an accountant's records belong to the corporation and should be intelligible to any other accountant). The system recommended here is a proven one, borrowed in part from that used in O. H. Lowry's laboratory. The general features of the system are described below.

1. Each record book has a unique number, which is issued chronologically.

2. When a notebook is filled, it is placed in the record library, which is located in the laboratory and accessible to all researchers.

3. The first five pages of each book are reserved for a table of contents.

4. The right-hand pages are used to record protocol, solution preparation, and results; that is, a log of laboratory activity.

5. The left-hand pages are for planning and interpretation. There it is appropriate to summarize a previous experiment (e.g., with a graph and a short statement), to outline the next experiment, to make a note of literature citations, to record calculations, etc.

6. Solution preparation should be recorded in a standardized, manner, thus:

1 M Tris(base) [121.1 g Tris(base) ∑ 91F-5002 qs 1000 ml H2O
[118.6 979

The above notation indicates the nominal concentration in the left margin. The supplier (Sigma) and lot number (91F-5002) are also recorded. (There are often significant differences in purity and impact of contaminants among suppliers and lots.) It is calculated that a 1 M solution would contain 121.1 g of Tris(base) per liter of solution. However, the researcher only weighs out approximately 121.1 g; to weight out exactly 121.1 g is inefficient. The lower numbers record the precise quantity weighed out and the final volume, adjusted accordingly. The bottle is labelled with the solution and concentration and the book and page numbers of the records documenting the preparation. A solution or reagent derived from this stock would be recorded, thus:

100 mM Tris-Cl [√10 ml 1 M Tris(base) 57/173
(pH 8.1) _ [√+ 0.2 ml 12 N HCl Mall KJAL
_ [√qs 100 ml H2O
[√electrode pH = 7.9

"57/173" indicates the book and page number documenting preparation of the 1 M Tris(base). Ingredients are added in order. The checks (_) are made as the ingredients are added.

Generally, solution concentrations are nominal. First, some reagents age (e.g., many enzymes lose activity during storage). Second, chemicals often are not supplied at absolute purity, and

some inaccuracy accrues from imprecision in mass and volume measurements. Third, many solutions are required in small amounts and are expensive. For example, 100 ml of 10 mM (+)abscisic acid could be prepared accurately, but would cost more than $100,000 US! On the other hand, the required amount, say 0.25 ml, is difficult to prepare. A compromise is to make the solution, not by diluting to a final volume, but by adding a certain volume of diluent, accounting in an approximate way for volume displacement by the solute, thus:

100 mM (+)ABA
√6.6 mg (+)ABA Supplier, Lot number
√+ 0.246 ml diluent

The total volume of this solution is approximately 0.25 ml, as 1 g ABA displaces about 0.6 ml of diluent.

In some cases, it is desirable to know precisely the concentration of reagent, even when the solution has necessarily been prepared imprecisely. Usually, the concentration is easy to measure. Thus, the original solution may be labelled 100 mM (+)ABA 57/175; after an accurate determination of the exact concentration, it is relabelled 97.4 mM (STD) (+)ABA 57/179.

7. Protocols may be written out in advance of their execution, but some system must be used to ensure they are followed, especially when time is of the essence. There are two simple ways to establish fidelity. One is to place a check by each task as it is accomplished. The second is accumulate "waste" (e.g., after a pipette is used, it can be set aside, in sequence, at the edge of the work area). A typical protocol may be recorded thus:

13 June 1983

Expt: $\Delta A_{340}/\Delta$time, + PGA + extract (= PGAL DH)

t_o	To Tubes 1–4, + 3 ml Assay Cocktail 57/128			
t_4 (at 0.3 minute intervals)	To Tubes 1, 2, + 30 µl extract-1 57/128			
	To Tubes 3, 4, + 30 µl extract-2 57/128			
A_{340} of Tubes	1	2	3	4
t_6 (at 0.3 minute intervals)	0.77	0.78	0.77	0.79
t_8	0.77	0.77	0.77	0.78
t_{10}	0.76	0.77	0.76	0.78
t_{12} (at 0.6 minute intervals)	To Tubes 1, 3, + 30 µl 200 mM PGA 57/114			
A_{340} of Tubes	1	2	3	4
t_{14} (at 0.3 minute intervals)	0.74	0.77	0.75	0.78
t_{16}	0.72	0.77	0.73	0.78
..... (etc.)				