

Chapter 14

Plant Hormones: Bioassay for Gibberellin

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Sandra Biroc received her B.A. in Biology from San Fernando Valley State College, California, in 1970 and her Ph.D. in Cell and Developmental Biology from The Johns Hopkins University, Maryland, 1975. She has taught courses at U.C. Davis and Cal. State Univ. Sacramento. She is presently lab coordinator in the Molecular, Cellular and Developmental Biology Department at the University of Colorado, Boulder, where she is in charge of the upper division lab courses in Cell Biology and Developmental Biology. In each course there are about 200 students that are separated into multiple sections of lab. She is deeply committed to providing quality laboratory instruction to undergraduates. Her most recent publication is a student manual of lab exercises for Junior and Senior level developmental biology students.

INTRODUCTION

The cereal grain seed germinates by first imbibing water then releasing hormones to begin digesting the protein and starch stored in the endosperm. Gibberellic acid is a hormone that is released by the embryo, travels to the aleurone layer and interacts with the genome of those cells to cause production of alpha- amylase. The enzyme travels to the endosperm which is broken down to simpler molecules such as sugars and amino acids. This bioassay uses wheat seeds with the embryo (wheat germ) removed. The hormone is added at various concentrations and allowed to incubate. After two days, the solution is assayed for the presence of reducing sugars with the Benedict's test. The color is compared to known concentrations of glucose to estimate amount of sugar produced. This is a highly enjoyable lab exercise because the results are clear cut, it is easy to do and the results are pretty colors. It is a very inexpensive exercise to prepare.

PRINCIPLES

Hormone Action

A **hormone** is a chemical substance that is produced in one place in an organism, is released into the body fluids, and has its effect on a **target tissue** in a place remote from the cells that produce it (Marx 1984). Hormones are in a sense chemical messengers that carry information from one type of cell to another. Not all cells are capable of responding to a given hormone, and not all sensitive cells respond the same way.

Animal Hormones

Animal hormones fall roughly into two categories: **peptide** hormones and **steroid** hormones. A peptide hormone interacts with its target cell by binding to a specific **receptor** located on the external cell membrane. The binding of hormone to receptor sets off a series of events that lead ultimately to the response. **Cyclic AMP** has been termed the "**second messenger**" because it can mimic the effect of some hormones (Sutherland, 1972). Other substances can mimic the effect, such as Ca^{2+} and polyphosphoinositides (see Marx, 1984 and Berridge, 1985) and are also sometimes called "second messengers."

The steroid hormones act by entering a cell (steroids are lipid soluble), binding to a receptor, either cytoplasmic or nuclear, and interacting with the chromatin to turn on a gene or set of genes. This phenomenon can be visualized by analyzing the puffing pattern of chromosomes from *Drosophila* salivary glands (Ashburner, et al., 1974).

Amplification

Both classes of hormones show **amplification of the signal**. For instance, for each molecule of insulin (a peptide hormone), there might be 10 molecules of adenyl cyclase activated, 100 molecules of cAMP, and 1000 molecules of kinase activated. And for estrogen (a steroid hormone) there might be a set of 10 genes turned on, which make 100 RNA molecules, which make 1000 protein molecules. This amplification process makes it possible to elicit a high cellular response from only a few hormone molecules.

Plant hormones

The plant hormones fall into five main categories: the auxins, the cytokinins, the gibberellins, growth inhibitors, and ethylene. Only one, gibberellic acid, will be studied.

Auxins are plant hormones produced by shoot meristems which help cause stem elongation in the cells just beneath the meristem. One example of an auxin is indole acetic acid (IAA). **Cytokinins** seem to be made in the roots and travel upward in the plant. The ratio of cytokinin to auxin determines whether a tissue grown in culture will be root tissue or shoot tissue; high cytokinin favors shoots, high auxin favors roots. The **gibberellins** promote stem elongation in intact plants. **Absciscic acid** and other growth inhibitors are responsible for dormancy and the release of fruit and leaves, **Ethylene** is a gas and is best known for its role in the ripening of fruit. Some chemical formulas for these plant hormones are shown in Figure 1.

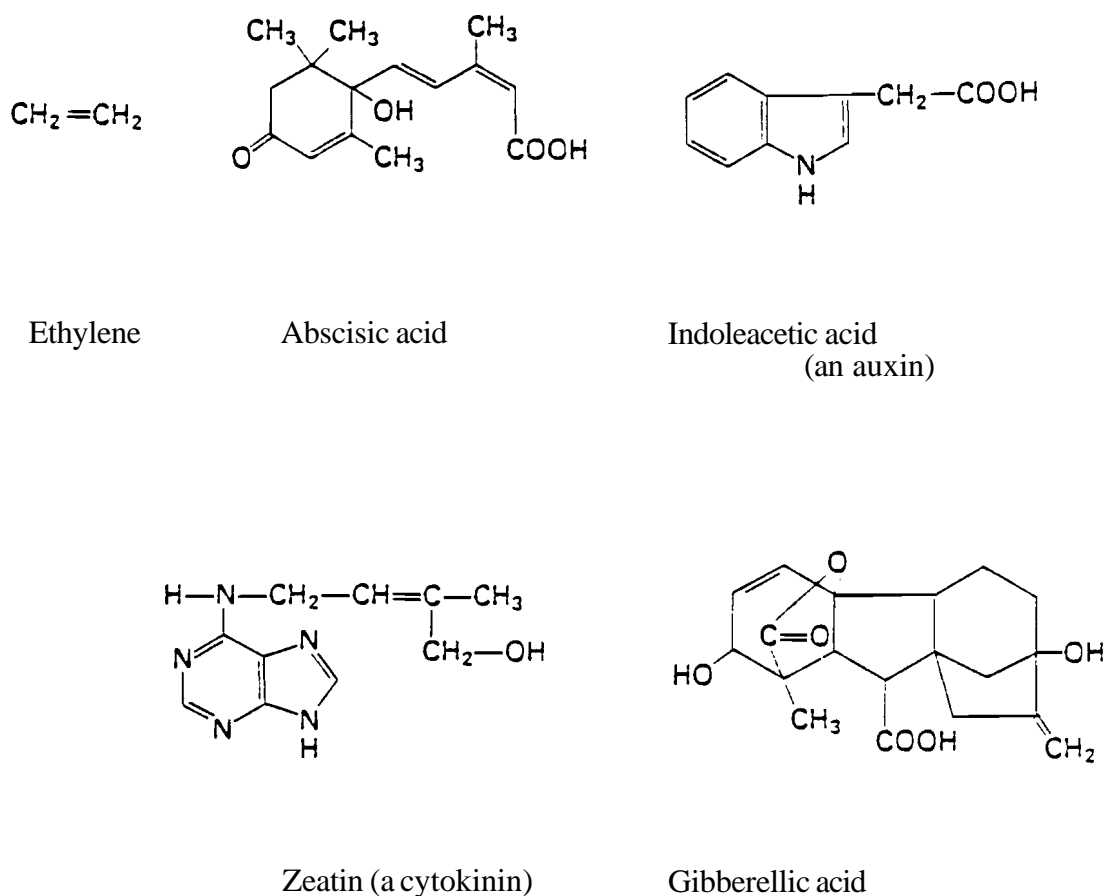
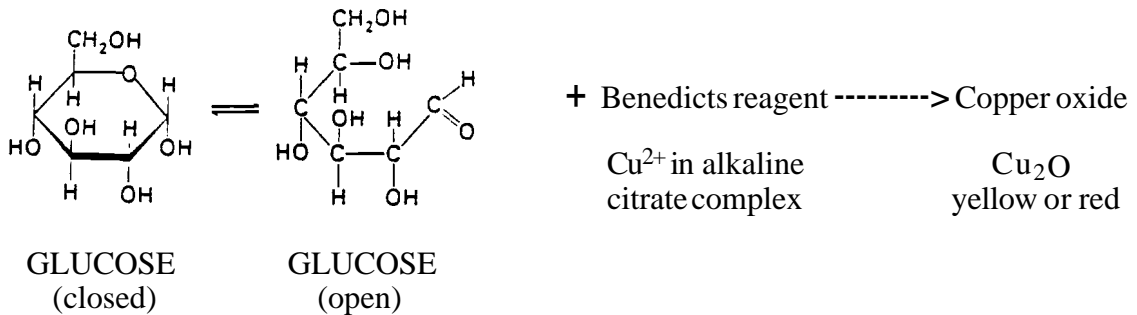


Figure 1. Some plant hormones.

The traditional **bioassay** for gibberellic acid makes use of its ability to induce starch breakdown in the endosperm of a barley seed (Fig. 2) whose embryo has been removed. In nature, a seed germinates by first imbibing water, which induces the embryo to release gibberellic acid. The **aleurone layer** (surrounding the endosperm) responds to the hormone stimulation by producing specific RNA molecules that code for the starch-digesting enzyme, **alpha-amylase**. Embryo-less half barley grains will merely imbibe water for several days in culture unless supplied with an exogenous source of gibberellic acid (Coombe et al, 1967). After only two days in the presence of the hormone, the alpha-amylase produced by the aleurone has digested enough endosperm starch (a large polymer of glucose residues) to be detected by a simple chemical test. Benedict's reagent (bluish, copper solution) turns yellow, orange, or red when heated in the presence of the free aldehyde of **reducing sugars** (such as glucose monomers):



This entire process can be inhibited by Actinomycin D, which blocks RNA synthesis, suggesting that gibberellin somehow causes specific genes to be turned on (see review by Galston and Davies, 1970).

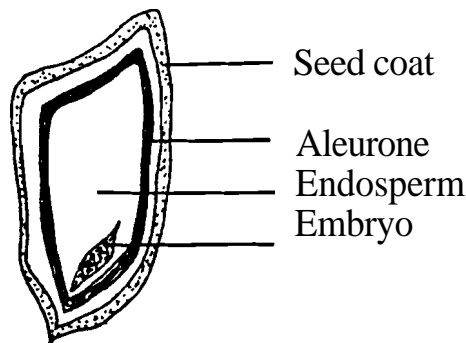


Figure 2. Longitudinal section of a barley grain.

Dose Response Curve

In any experiment where a drug or hormone is being used, it is important to determine the optimal dosage to use. A dose response curve is constructed by varying the concentration of drug or hormone and observing the degree of response the system gives (Fig. 3).

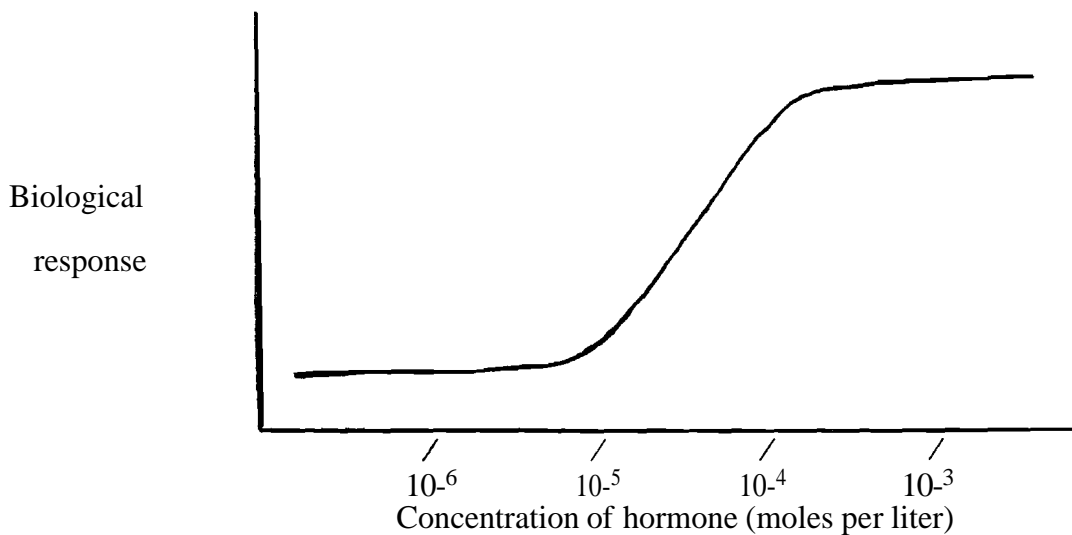


Figure 3. Dose response curve. Varying concentrations of hormone are tested and the response observed. The data are plotted with concentration on the abscissa and response on the ordinate.

PROCEDURE

Starch breakdown in embryo-less wheat half-seeds induced by gibberellic acid. The dose response curve.

Session #1

1. Select 10 wheat seeds and place them on a sheet of dental wax. Using a sharp razor blade, cut each seed in half so that you have a half containing the embryo and a half that does not. Discard the half containing the embryo. Slice the remaining half down the middle and place both "quarters" into a 100 ml beaker.
2. Prepare three more batches of 10 seeds and place them in a second, third and fourth beaker.
3. Surface sterilize the embryo-less half seeds by treating as follows:
 - a) Add 10 ml of half-strength Clorox. Swirl, then allow to sit 10 minutes. Aspirate off the liquid using a sterile pasteur pipette.
 - b) Add 10 ml sterile 0.01 N HCl. Swirl. Aspirate.
 - c) Add 10 ml sterile water. Swirl. Aspirate.
 - d) Add 10 ml sterile water. Swirl. Aspirate.
 - e) Sterilize a spatula by wiping it with 70% ethanol. Use this spatula to scoop the seeds into each of four sterile, 50 mm petri plates.
4. Add 10 ml of sterile acetate buffer to each plate.

Prepare two serial 100-fold dilutions of the hormone. Work sterilely.

- a) Pipette 1 ml of acetate buffer into each of two sterile test tubes. Label these A (1:100) and B (1:10,000).
 - b) Remove 10 μ l of hormone from the 1 mM stock solution and add it to the first tube. Swirl to mix.
 - c) Remove 10 μ l from A and add it to B. Swirl to mix.
6. Add the hormone dilutions to the petri plates.
- a) Add 10 μ l of 1 mM gibberellic solution to one plate for a concentration of 1000 nanomolar.
 - b) Add 10 μ l of "A" dilution to a second plate for a concentration of 10 nanomolar
 - c) Add 10 μ l of "B" dilution to a third plate for a concentration of 0.1 nanomolar.
 - d) Make no additions to the fourth (control) plate.

7. Label four test tubes as:

day 0, 1000nM
day 0, 10nM
day 0, 0.1 nM
day 0, control

Remove 1 ml from each plate and pipette into the waiting test tubes. Store these tubes in the freezer. These are the "zero-time controls."

8. Tape the plates together, four high, label the tape clearly and place these in the box your TA has set aside for your section. The box will remain at room temperature for two days and then will be placed in the refrigerator until the section meets again.

Session #2

[The lab instructor will place your plates in the refrigerator after two days of incubation to slow down any bacterial or fungal growth. Ideally, these should be assayed after two days of incubation.]

1. Examine the three plates for evidence of softening of the endosperm (starchy) tissue. Jot down the observations in your notebook.
2. Prepare to test the supernatant liquid for the presence of reducing sugars.
 - a) Retrieve the zero-time samples from the freezer. Thaw.
 - b) Remove 1 ml from each petri plate and place the liquid in separate 9 ml test tubes. Label these with a permanent (not water soluble) marking pen.
3. Prepare a standard curve of glucose
 - a) Set out 5 test tubes, label these 1, 2, 3, 4 and 5.
 - b) Add 0.9 ml acetate buffer to tubes 2, 3, 4 and 5.
 - c) Add 1 ml stock dextrose to tube #1.
 - d) Add 0.1 ml stock dextrose to tube #2. Swirl to mix.
 - e) Add 0.1 ml from tube #2 to tube #3. Swirl to mix.
 - f) Repeat step "e" until all five dilutions are made.

4. The color reaction

- a) Prepare a boiling water bath using a 250 ml beaker filled one third full of water and a few boiling chips.
- b) Add 2 drops of Benedict's solution to all thirteen test tubes. Swirl to mix. Place the tubes in the water bath for 2-3 minutes.
- c) Remove the tubes, swirl, and jot down the observations in your notebook.

<u>Tube #</u>	<u>Sample</u>	<u>Color Observed</u>
1	10% dextrose	_____
2	1% dextrose	_____
3	0.1 % dextrose	_____
4	0.01% dextrose	_____
5	0.001% dextrose	_____
6	Control plate, day 0	_____
7	1000 nanomolar gibberellin, day 0	_____
8	10 nanomolar gibberellin, day 0	_____
9	0.1 nanomolar gibberellin, day 0	_____
10	Control plate, day 2	_____
11	1000 nanomolar gibberellin, day 2	_____
12	10 nanomolar gibberellin, day 2	_____
13	0.1 nanomolar gibberellin, day 2	_____

VOCABULARY

hormone, target tissue, responding cell, peptide hormone, steroid hormone, receptor, second messenger, amplification of the signal, auxin, cytokinin, gibberellin, aleurone layer, alpha-amylase, reducing sugar.

QUESTIONS

- 1. Animal hormones fall into two major classes. What are they? What is the difference in their mode of action within the cell?
- 2. Explain what is meant by a "second messenger" and give an example.
- 3. Hormones occur in very low concentrations and yet can elicit very dramatic cellular responses. Give an example of signal amplification.
- 4. Name two plant hormones other than gibberellic acid and describe what each does.
- 5. In a natural seed, what is the source of gibberellic acid? Why did you have to supply gibberellic acid to the experimental seeds?
- 6. Explain what is meant by a "reducing sugar." Chemically, explain why the control plate did not show any reducing sugars present, but the treated plate did. Where does the sugar come from?

7. Plant hormones fall into five categories (according to some sources).
- Name three of the five. At least name one major hormone from three of the five categories.
 - What is the name of the plant hormone that you added to the petri plates of embryo-less wheat half-seeds?
8. A wheat seed undergoes a series of cellular changes in response to imbibing water. In the natural situation (not a laboratory experiment) what is the source of the hormone? What tissue does it act on? What molecule does that tissue make in response to the hormone? What does that molecule do to the starch?

source of hormone _____
 responding tissue _____
 molecule produced _____
 action on starch _____

DIRECTIONS TO THE PREPARATOR

Biologicals: Barley, wheat or oat seeds. Purchase from a local seed store.

Equipment: test tubes, sterile 60 mm petri plates, alcohol lamp, spatula, razor blade, dental wax, pipettes, boiling water bath, heating plates, microliter capillary pipettes (10 μ l) or automatic pipettor set for 10 μ l and sterile tips, permanent marking pen.

Solutions: Clorox, 0.01N HCl (autoclaved), acetate buffer (autoclaved), distilled water (autoclaved), gibberellic acid (1 mM, sterile), Benedict's reagent, 70% alcohol for sterilizing.

Benedict's reagent;

- Add 173 gm Na citrate and 100gm Na_2CO_3 to 800 ml d- H_2O . Warm and stir to dissolve.
- Cool and filter.
- Add d- H_2O to 850 ml.
- Dissolve 17.3 gm CuSO_4 in 100 ml d- H_2O . Add SLOWLY with stirring to 1 liter.
- Add d- H_2O to 1 liter.

Gibberellic acid solution: (1 mM)

35 mg GA3 (Sigma) plus 10 ml 95% ethanol. Dissolves completely in 5 minutes. Add 90 ml d-water. No cloudiness evident. Sterilize by filtration. If refrigerated promptly, can be stored for years.

Acetate buffer:

	<u>M.W.</u>	<u>gm/liter</u>	<u>molarity</u>
sodium acetate	86	0.172	2 mM
calcium chloride	111	2.2	20 mM

Add salts to 800 ml d-water, adjust pH to 4.2 with 0.1 N HCl; bring volume to 1 liter; autoclave 20 minutes. cool. store at room temp.

REFERENCES

- Alberts B. et al. (1983). *Molecular Biology of the Cell*. Garland. New York, pp. 510-516.
- Altman, J. (1988). Ins and outs of cell signalling. *Nature* 331:119-120.
- Ashburner, M. Chihara, C., Meltzer, P., and G. Richards. (1974). Temporal control of puffing activity in polytene chromosomes. Cold Spring Harbor Symp. *Quant. Biol.* 38:655-662.
- Berridge, M.J. (1985). The molecular basis of communication within the cell. *Sci. Am.* Oct. pp 142-152.
- Biroc, S. L. (1986). *Developmental Biology; A Laboratory Manual with Readings*. Macmillan. New York.
- Conn, E.E. and P.K. Stumpf (1972). *Outlines of Biochemistry*. John Wiley & Sons. New York.
- Coombe, B.G., Cohen, D. and L.G. Paleg (1967). Barley endosperm bioassay for gibberellins. I. Parameters of the response system. *Plant Physiol.* 42:105-112.
- Curtis, H. (1979). *Biology*. 3rd ed. Worth Publishers. New York. pp, 491-507.
- Darnell, Lodish Baltimore (1986). *Molecular Cell Biology*. Scientific American Books. Freeman. New York.
- Galston, A.W. and Davies, P.J. (1970). *Control mechanisms in plant development*. Prentice-Hall, Inc. Englewood Cliffs, New Jersey.
- Higgins, T.J.V., Zwar, J.A. and J.V. Jacobsen (1976). *Nature* 260:166.
- Jacobsen, J.V. and L.R. Beach (1985). Control of transcription of α -amylase and rRNA genes in barley aleurone protoplasts by gibberellin and abscisic acid. *Nature* 316:275-277.
- Lang, A. (1957). The effect of gibberellin upon flower formation. *Proc. Natl. Acad. Sci.* 43:709-717.
- Marx, J.L. (1984). A new view of receptor action. *Science* 224:271.
- Mozer, T.J. (1980). Control of Protein Synthesis in barley aleurone layers by the plant hormones gibberellic acid and abscisic acid. *Cell* 20:479-485.
- Wittwer, S.H., Bukovac, M.J., Sell, H.M. and L.E. Weller (1957). Some effects of gibberellin on flowering and fruit setting. *Plant Physiol.* 32:39-41.