EXPERIMENT No. 1

Isolation of a fluorescent protein: Stability studies on phycocyanin

The phycobiliproteins are proteins with linear tetrapyrrole prosthetic groups (bilins) that, in their functional state, are covalently linked to specific cysteine residues of the proteins. These proteins are found in cyanobacteria (blue-green algae). The phycobiliproteins can be divided into three main classes depending on absorption properties: phycoerythrins (PE, λ_{max} 540–570 nm), phycocyanins (PC, λ_{max} 610–620 nm), and allophycocyanins (APC, λ_{max} 650–655 nm).

The pigment, which gives Spirulina its blue colour, is phycocyanin, found in concentrations of about 7 percent, compared to the 1 percent chlorophyll content most commonly found. The protein to be isolated is a colored protein called phycocyanin. This protein has a very simple function; it acts to hold the pigment molecule in a specific conformation or position. Phycocyanin will be isolated from *Spirulina* cells. Phycocyanin is related to the human pigment bilirubin, which is important to healthy liver function and digestion of amino acids.

In the experiment, we will see what kind of molecules make the protein structure more stable and what makes the protein unfold. The protein will be a dark blue solution when it is properly folded and a pale blue or colorless solution when unfolded. When the protein isn't properly folded, the protein molecules tend to aggregate together and precipitate out of solution making it look milky. Also, the folded protein gives off a red fluorescence glow. If the protein is unfolded, the red glow disappears along with the blue color. The protein fluoresces when it is folded properly since it is used to collect photons for photosynthesis. Since the protein has been isolated from the other photosynthetic proteins, the photons escape as light (i.e. red fluorescence) since they are not taken up in any photosystem reaction (photosystem II in this case). Therefore, the red fluorescence tells us the protein is working properly. The lack of red fluorescence means the protein has been adversely affected, i.e. unfolded.

A. Protein Isolation

1. Select one of the Spirulina capsules provided and pour the contents into a tared weighing dish on an electronic top loading balance. Record the mass and place the bacteria into a mortar.
2. Weigh approximately the same mass of fine silica and add it to the bacteria in the mortar. Grind the bacteria and silica together for a few minutes. Ensure maximum breaking of the cell walls by grinding the bacteria against the side as well as the bottom of the mortar. The mixture should have smooth texture after grinding.
3. **Add approx**. **7-10 mL of 0.1 M sodium phosphate buffer at pH 7 to the spirulina mixture in a single test tube.** Make sure all the silica/bacteria mixture is suspended in the buffer solution.
4. Mix the solutions with a glass stirring rod, then centrifuge for two minutes at 10000 rpm.
5. After centrifugation, carefully pour off the clear liquid from the silica and unbroken cells at the bottom of the test tube into a clean test tube. Discard the solid left in the tube.
6. Filter the contents of the test tube into a small conical flask. The protein solution in the Erlenmeyer flask should be clear and dark blue colored.

B. Determination of amount of phycobiliproteins

The amounts of PE, PC and APC in the different extracts and biliprotein containing solutions can be calculated from measurements of the absorbance at 565, 620 and 650 nm using the following equations.

$$\text{R-PC}(\text{mg ml}^{-1}) = \frac{(\text{OD}_{620\,\text{nm}} - 0.7\text{OD}_{650\,\text{nm}})}{7.38}$$

$$\text{APC}(\text{mg ml}^{-1}) = \frac{(\text{OD}_{650\,\text{nm}} - 0.19\text{OD}_{620\,\text{nm}})}{5.65}$$

$$\text{B-PE}(\text{mg ml}^{-1}) = \frac{(\text{OD}_{565\,\text{nm}} - 2.8[\text{R} - \text{PC}] - 1.34[\text{APC}])}{12.7}$$

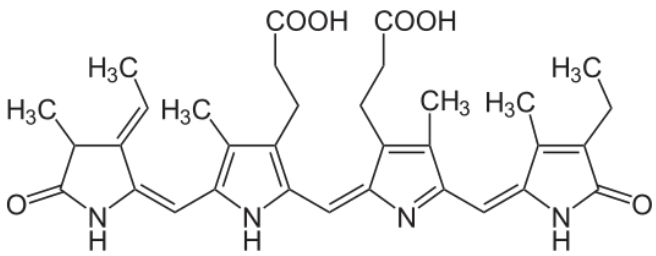
C. Intermolecular forces in proteins

Use 0.5 ml of protein solution added to 2.5 ml of the following:

1. 10% Sodium Dodecyl Sulfate (SDS)
2. 0.10 M Sodium Chloride
3. Acetone
4. 6.0 M Urea
5. 1.0 M Sucrose
6. 1.0 M HCl
7. 1.0 M NaOH
8. Solution (protein in buffer) kept in boiling water for two minutes
9. Solution (protein in buffer) kept on ice for two minutes

Answer the following questions for each solution and record your results in tabular form explain the effect in each case.

What is the **color of the solution**?
Is the solution **clear or cloudy**?
Is the solution **emitting red fluorescence**?
Is the protein **folded or unfolded**?



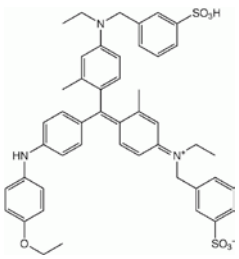
Phycocyanobilin

Expt. No. 2a: Bradford Protein Assay

Principle

The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford 1976). The dye exists in three forms: cationic (red), neutral (green), and anionic (blue) (Compton and Jones 1985). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (A_{max} = 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (A_{max} = 595 nm) (Reisner et al. 1975, Fazekes de St. Groth et al. 1963, Sedmack and Grossberg 1977). It is this blue protein-dye form that is detected at 595 nm in the assay using a spectrophotometer or microplate reader. Work with synthetic polyamino acids indicates that Coomassie Brilliant Blue G-250 dye binds primarily to basic (especially arginine) and aromatic amino acid residues (Compton and Jones 1985). Spector (1978) found that the extinction coefficient of a dye-albumin complex solution was constant over a 10-fold concentration range. Thus, Beer's law may be applied for accurate quantification of protein by selecting an appropriate ratio of dye volume to sample concentration. Certain chemical-protein and chemical-dye interactions interfere with the assay. Interference from non-protein compounds is due to their ability to shift the equilibrium levels of the dye among the three colored species. Known sources of interference, such as some detergents, flavonoids, and basic protein buffers, stabilize the green neutral dye species by direct binding or by shifting the pH (Compton and Jones 1985, Fanger 1987).

Nevertheless, many chemical reagents do not directly affect the development of dye color when used in the standard protocol.



Background of Colour Changes: The colour of the two dyes depends on the acidity of the solution and on its binding status to amino acids or peptides. At a pH of less than 0 the dye has a **red** colour with an absorption maximum at a wavelength of 470 nm. At a pH of around 1 the dye is **green** with an absorption maximum at 620 nm while above pH 2 the dye is bright **blue** with a maximum at 595 nm. The different colours result from the differently charged states of the dye molecule, corresponding to the amount of positive charges at the three nitrogen atoms present, while the two sulfonic acid groups are normally always negatively charged.

- At a pH of around zero, all three nitrogen atoms are positively charged, thus the dye will be a cation with an overall charge of +1, being in the **red** form.
- In the **green** form (pH approx. 1) the dye will have no net overall charge (+2 and -2).
- At pH of 2 and more, up to the neutral pH, only one nitrogen atom carries a positive charge and the dye molecule is a **blue** anion with an overall charge of -1.
- Under alkaline conditions, the final proton is lost and the dye becomes **pink** in colour. This state, however, is of no relevance in biochemical assays.

Materials

1. BSA (1 mg/ml) solution
2. Commassie blue 1x dye solution
3. Protein with unknown concentration

Standard Protocol

1. The standard protocol can be performed in three different formats, 5 ml and a 1 ml cuvette assay, and a 250 µl microplate assay. The linear range of these assays for BSA is 125–1,000 µg/ml, whereas with gamma-globulin the linear range is 125–1,500 µg/ml.
2. Remove the 1x dye reagent from 4 °C storage and let it warm to ambient temperature. Invert the 1x dye reagent a few times before use.
3. If 2 mg/ml BSA or 2 mg/ml gamma-globulin standard is used, refer to the tables in the appendix as a guide for diluting the protein standard. For the diluent, use the same buffer as in the samples. Protein solutions are normally assayed in duplicate or triplicate. For convenience, blank samples (0 µg/ml) should be made using water and dye reagent.
4. Pipette each standard and unknown sample solution into separate clean test tubes or microplate wells (the 1 ml assay may be performed in disposable cuvettes). Add the 1x dye reagent to each tube (or cuvette) and vortex (or invert).

Assay	**Volume of Standard and Sample**	**Volume of 1x Dye Reagent**
5 ml	100 µl	5 ml
1 ml	20 µl	1 ml

5. Incubate at room temperature for at least 5 min. Samples should not be incubated longer than 1 hr at room temperature.
6. Set the spectrophotometer to 595 nm. Zero the instrument with the blank sample (not required for microplate readers). Measure the absorbance of the standards and unknown samples.

Data Analysis

1. If the spectrophotometer or microplate reader was not zeroed with the blank, then average the blank values and subtracts the average blank value from the standard and unknown sample values.
2. Create a standard curve by plotting the 595 nm values (y-axis) versus their concentration in µg/ml (x-axis). Determine the unknown sample concentration using the standard curve. If the samples were diluted, adjust the final concentration of the unknown samples by multiplying with the dilution factor used.

References

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2. Compton SJ and Jones CG, Mechanism of dye response and interference in the Bradford protein assay, Anal Biochem 151, 369–374 (1985)
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Expt. No. 2b: Protein estimation protocol (Lowry assay method)

Working principle

The method combines the reactions of cupric ions with the peptide bonds under alkaline conditions with the oxidation of aromatic protein residues. The Lowry method is best used with protein concentrations of 0.01-1.0 mg/mL and is based on the reaction of Cu^{+}, produced by the oxidation of peptide bonds, with Folin-Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in phenol) in the Folin-Ciocalteu reaction. The reaction mechanism is not well understood, but involves reduction of the Folin reagent and oxidation of aromatic residues (mainly tryptophan, also tyrosine). This is poorly understood but in essence phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The concentration of the reduced Folin reagent is measured by absorbance at 750 nm. As a result, the total concentration of protein in the sample can be deduced from the concentration of Trp and Tyr residues that reduce the Folin reagent.

The disadvantage of this method is the long incubation time and there are often interferences with commonly used buffers. This method is also subject to protein-to-protein variation due to the correlation of colour intensity dependent on the content of tyrosine and tryptophan in the protein.

Reagents and solutions:

Solution A: Dissolve 0.4 g of sodium tartarate (Mw = 230.1) and 20 g Na_2CO_3 in 100 mL of 1N NaOH, dilute with DIW to 200 mL.

Solution B: Alkaline copper-tartrate solution: Dissolve 2 g Na-K-tartrate (Rochelle salt, Mw = 282.2) and 1 g $CuSO_4,5H_2O$ in 10 mL 1 N NaOH.

Solution C: Diluted FCR solution (Folin-Ciocalteu reagent): 1 part FCR diluted with 15 parts of DIW.

Solution D: Known protein solution and unknown protein solution

Procedure: Protein solution should contain 4-40 µg of protein. With the known supplied protein solution make 10-12 samples (eppendorf tube) of different concentration in the above range. Add 0.6 ml of DIW and 0.6 mL of solution A to each sample and incubate for 20 min at RT. Then add 66 µl of solution B to each tube and let it stand for another 10 min. Then add 2 mL of solution C and mix well, keep it for 30 min and record the absorption at 750 nm. Draw the calibration curve (Abs vs c) and determine the unknown protein concentration from the calibration curve.

Notes:

1. If the sample is available as a precipitate, then dissolve the precipitate in 2N NaOH.

2. The reaction is very pH-dependent, and it is therefore important to maintain the pH between 10 and 10.5. Take care, therefore, when analyzing samples that are in strong buffer outside this range.

3. The incubation period is not critical and can vary from 10 min to several hours without affecting the final absorbance.

4. The Vortex step is critical for obtaining reproducible results. The Folin reagent is only reactive for a short time under these alkaline conditions, being unstable in alkali, and great care should therefore be taken to ensure thorough mixing.

5. The assay is not linear at higher concentrations. Ensure, therefore, that you are analyzing your sample on the linear portion of the calibration curve.

6. A set of standards is needed with each group of assays, preferably in duplicate. Duplicate or triplicate unknowns are recommended.

7. One disadvantage of the Lowry method is the fact that a range of substances interfere with this assay, including buffers, drugs, nucleic acids, and sugars. In many cases, the effects of these agents can be minimized by diluting them out, assuming that the protein concentration is sufficiently high to still be detected after dilution. When interfering compounds are involved, it is, of course, important to run an appropriate blank. Interference caused by detergents, sucrose, and EDTA can be eliminated by the addition of SDS. The best alternative in this case is to do Lowry-TCA

8. Modifications to this basic assay have been reported that increase the sensitivity of the reaction. If the Folin reagent is added in two portions, vortexing between each addition, a 20% increase in sensitivity is achieved. The addition of dithiothreitol 3 min after the addition of the Folin reagent increases the sensitivity by 50%.

9. The amount of color produced in this assay by any given protein (or mixture of proteins) is dependent on the amino acid composition of the protein(s) (see Introduction). Therefore, two different proteins, each for example at concentrations of 1 mg/mL, can give different color yields in this assay. It must be appreciated, therefore, that using BSA (or any other protein for that matter) as a standard only gives an approximate measure of the protein concentration. The only time when this method gives an absolute value for protein concentration is when the protein being analyzed is also used to construct the standard curve. The most accurate way to determine the concentration of any protein solution is amino acid analysis.

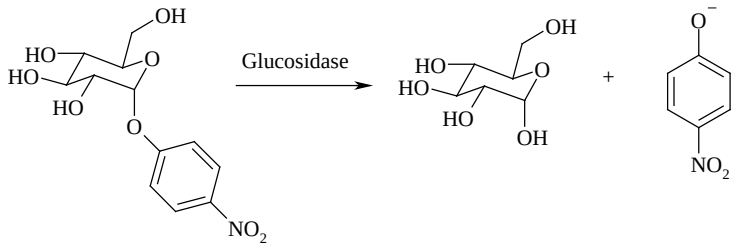
Expt. No. 3a: Enzymatic Assay of α-*GLUCOSIDASE* (EC 3.2.1.20) by using *p*-Nitrophenyl α-D-Glucoside as a Substrate

PRINCIPLE:

p-Nitrophenyl α-D-Glucoside $\xrightarrow{\text{αGlucosidase}}$ α-D-Glucose + p-Nitrophenol

CONDITIONS: T = 37°C, pH = 6.8, A_{400}nm, Light path = 1 cm

METHOD: Spectrophotometric Stop Rate Determination



REAGENTS:

A. 67 mM Potassium Phosphate Buffer, pH 6.8 at 37°C (Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Adjust to pH 6.8 at 37°C with 1 M NaOH. **PREPARE FRESH.**)

B. 3 mM Glutathione, Reduced Solution (GSH). (Prepare 10 ml in deionized water using Glutathione, Free Acid, Reduced Form, **PREPARE FRESH**.)

C. 10 mM 'Glucoside Solution (PNP-Gluc). (Prepare 10 ml in deionized water using p-Nitrophenyl α-D-Glucopyranoside,

D. 100 mM Sodium Carbonate Solution, (NaCarb) (Prepare 50 ml in deionized water using Sodium Carbonate, Anhydrous)

E. α-Glucosidase Enzyme Solution (Immediately before use, prepare a solution containing 0.15 - 0.3 unit/ml of α-Glucosidase in cold deionized water.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable containers:

	Test	**Blank**
Deionized Water	------	0.20
Reagent A (Buffer)	5.00	5.00
Reagent B (GSH)	0.20	0.20
Reagent E (Enzyme Solution)	0.20	------
Mix by inversion and equilibrate to 37°C. Then add:		
Reagent C (PNP-Gluc)	0.50	0.50

Immediately mix by inversion and incubate for exactly 20 minutes at 37°C.

Pipette (in milliliters) the following reagents into suitable containers:

Test Solution	2.00	------
Blank Solution	------	2.00
Reagent D (NaCarb)	8.00	8.00

Mix by inversion and transfer the solutions to suitable cuvettes. Record the A_{400}nm for both the Test and blank using a suitable spectrophotometer.

CALCULATIONS:

Units/ml enzyme = (A_{400}nm Test - A_{400}nm Blank)(10)(5.9)(df) / (18.3)(20)(2)(0.2)

5.9 = Volume (in milliliters) of reaction mixture

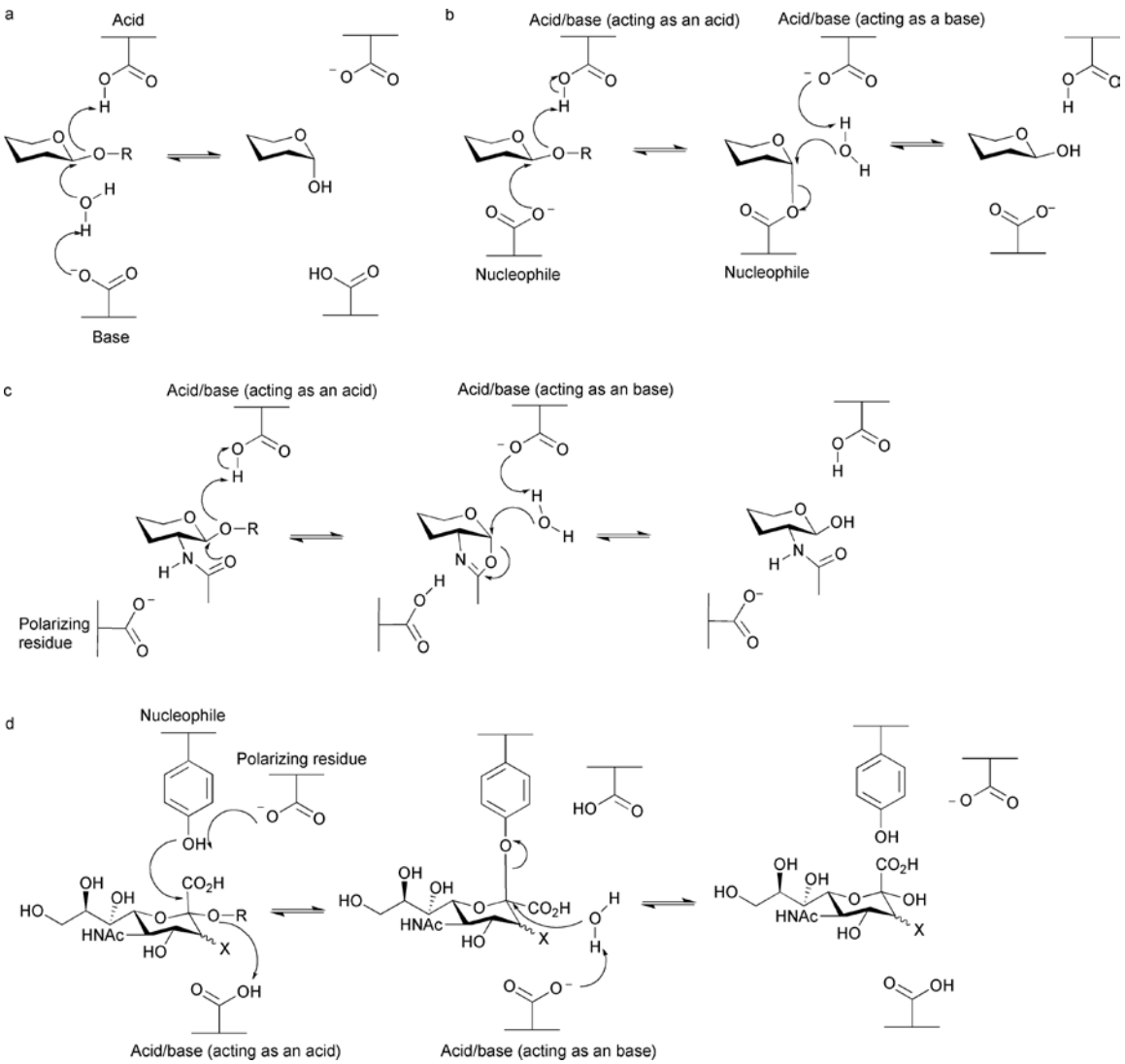
df = Dilution factor
18.3 = Millimolar extinction coefficient of p-Nitrophenol at 400 nm
20 = Time (in minutes) of the assay
10 = Volume (in milliliters) of Colorimetric Determination
2 = Volume (in milliliters) of reaction mix used in the colorimetric determination

CALCULATIONS:
Units/mg solid = units/ml enzyme / mg solid/ml enzyme

Units/mg protein = units/ml enzyme / mg protein/ml enzyme

UNIT DEFINITION:
One unit will liberate 1.0 µ mole of D-glucose from p-nitrophenyl α-D-glucoside per minute at pH 6.8 at 37°C.

FINAL ASSAY CONCENTRATIONS:
In a 5.90 ml reaction mix, the final concentrations are 57 mM potassium phosphate, 0.01 mM glutathione, 0.85 mM p-nitrophenyl α-D-glucoside and 0.03-0.06 unit α-glucosidase.



Glycosidase mechanisms for hydrolysis: (a) 'Classical' mechanism for inversion of stereochemistry. (b) 'Classical' mechanism for retention of stereochemistry. (c, d) Substrate-assisted mechanism.

Expt. No. 3b: Peroxidase assay (EC 1.11.1.7) [donor: hydrogen peroxide oxidoreductase]

The enzyme, usually prepared from horseradish, is a glycoprotein with a molecular mass of 44KD. It is related to catalase, carrying a protohematin IX as a prosthetic group. It is specific for the acceptor (H_2O_2), but reacts with numerous donor substrates, which may be used for the enzyme assay. Peroxidase can be used for treatment of industrial waste waters. For example, phenols, which are important pollutants, can be removed by enzyme-catalyzed polymerization using horseradish peroxidase. Thus phenols are oxidized to phenoxy radicals, which participate in reactions where polymers and oligomers are produced that are less toxic than phenols. It also can be used to convert toxic materials into less harmful substances.

$$ROOR' + \text{electron donor } (2\ e^-) + 2H^+ \rightarrow ROH + R'OH$$

Most reactions catalysed by HRP-C and other horseradish peroxidase isoenzymes can be expressed by the following equation, in which AH2 and $AH^{\bullet}$ represent a reducing substrate and its radical product, respectively. Typical reducing substrates include aromatic phenols, phenolic acids, indoles, amines and sulfonates.

$$H_2O_2 + 2AH_2 \xrightarrow{HRP\ C} 2H_2O + 2AH^{\bullet}$$

Guaiacol assay: Based on that of Bergmeyer in which the rate of decomposition of hydrogen peroxide by peroxidase, with guaiacol as hydrogen donor, is determined by measuring the rate of colour development spectrophotometrically at 436 nm and at 25°C.

$$4\ \text{Guaiacol} + 4H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Tetraguaiacol} + 8H_2O$$

Unit Definition

The amount of enzyme which catalyses the conversion of one micromole of hydrogen peroxide per minute at 25°C.

Reagents

A 0.1 M Potassium Phosphate Buffer pH 7.0

Dissolve 0.53g KH_2PO_4 [MM: 136.09] and 1.06g K_2HPO_4 [MM: 174.18] in distilled H_2O, check pH to 7.0 and dilute to 100 ml. Store diluents on ice and equilibrate buffer at 25°C.

B 0.018 M Guaiacol

Dissolve 22.3 mg guaiacol (MM: 124.14) in 10 ml distilled water. Store on ice and prepare fresh daily.

C Substrate:

Dilute 0.1 ml 30% hydrogen peroxide with distilled water to 120 ml and adjust A_{240} in 1 cm light path to 0.4 to 0.41 versus distilled H_2O. Store the solution on ice and prepare fresh daily.

D Enzyme Solution:

Dissolve 5 mg enzyme / ml 0.1M ice cold potassium phosphate buffer pH 7.0 (refer reagent 3A above). Immediately before assay, dilute to yield approximately 0.2 units / ml ice cold buffer. (Approximately 0.040 to 0.045 ΔA_{436}/minute.)

Procedure

Temperature = 25°C
Wavelength = 436 nm
Light Path = 1 cm

Into a 1cm quartz cell, pipette the following:

Buffer	2.80 ml
Guaiacol	0.05 ml
Substrate	0.05 ml
Equilibrate at 25°C and monitor ΔA/minute	
Enzyme at zero time	0.10 ml
Total reaction volume	3.00 ml

Record the rate of increase in absorbance at 436 nm using the linear portion of the curve after the initial lag phase.

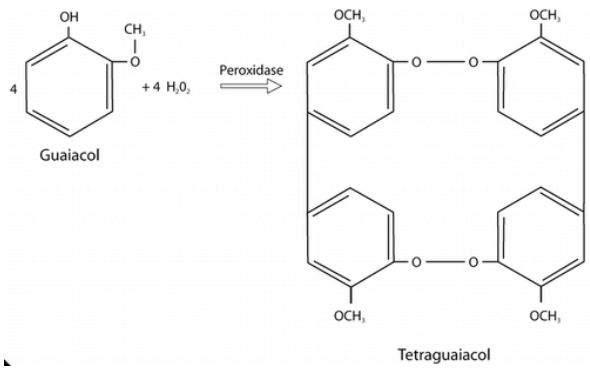
Calculation

$$\textbf{Volume activity (U / ml)} = \frac{\Delta A_{436}/\text{min} \times 4 \times Vt \times \text{dilution factor}}{\varepsilon \times Vs}$$

Where

Vt = final volume of reaction mixture (ml) = 3.00
Vs = sample volume (ml) = 0.1
ε = micromolar extinction co-efficient of tetraguaiacol (cm^2/micro mol) = 25.5
4 = derived from unit definition & principle

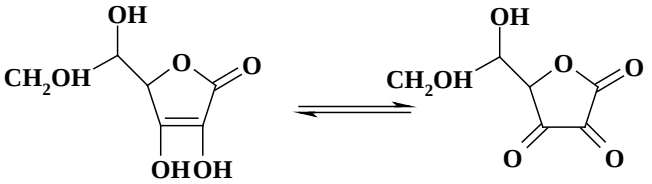
$$\textbf{Weight activity (U/mg material)} = \frac{\text{U/ml}}{\text{mg enzyme/ml original solution}}$$



Expt. No. 4a: Vitamin C Estimation

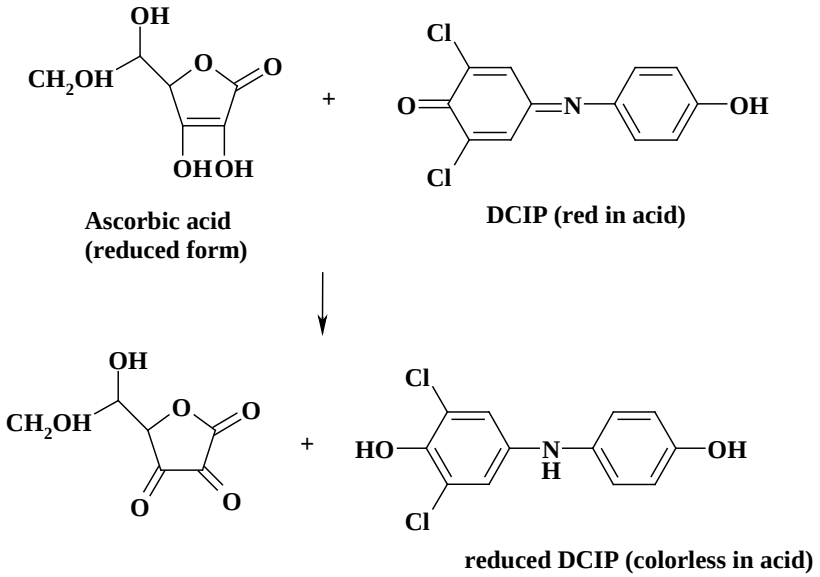
Working Principle:

Ascorbic acid is widely distributed in nature, but it occurs in especially high concentration in citrus fruits and green plants such as spinach and green peppers. Chemically ascorbic acid is water soluble, slightly acidic carbohydrate that exists in an oxidized or reduced form.



Ascorbic acid (reduced form) ⇌ **Dehydroascorbic acid (oxidized form)**

Because of the clinical significance of vitamin C, it is essential to be able to detect and quantify its presence in various biological samples. Ascorbic acid may be assayed by titration with iodine, reaction with 2,4-DNP or titration with a redox indicator, 2,6-dichlorophenolindophenol (DCIP) in acid solution. The latter method will be used in this experiment because it is reasonably accurate, rapid and convenient. Samples for analysis often contain traces of other compounds, in addition to ascorbic acid, that reduce DCIP. One way to minimize the interference of other substances is to analyze two identical aliquots of the sample. One aliquot is titrated directly and the total content of all reducing substances present is determined. The second aliquot is treated with *ascorbic acid oxidase* to destroy ascorbic acid and then titrated with DCIP. The second titration allows determination of reducing substances other than ascorbic acid. A second way to reduce the effect of other reducing substances is to perform the titration in the pH range 1 to 3, the interfering agents react very slowly with DCIP under this condition.



Ascorbic acid (reduced form) + **DCIP (red in acid)** → **reduced DCIP (colorless in acid)**

Materials and supplies:

1. Fruit juices (Lime)
2. Vitamin C tablets
3. Metaphosphoric acid/ acetic acid solution, 4%
4. Unknown sample in Metaphosphoric acid/ acetic acid solution (4%)
5. Standard Vitamin C solution in Metaphosphoric acid/ acetic acid solution (4%), 0.5 mg/ml
6. DCIP solution in DIW, 25 mg/100 ml
7. Ascorbic acid oxidase, lyophilized powder
8. Microburrete 10 mL
9. Filter paper
10. Glass funnel

Estimation of Vitamin C

A. Standard ascorbic acid solution:

1. Fill a microburrete with DCIP solution.
2. Transfer 1.0 mL of the ascorbic acid standard solution to a 50 mL conical flask.
3. Titrate by rapid, dropwise addition of DCIP solution while mixing the contents of the flask until a distinct rose-pink color persists for 15-20 seconds.
4. Repeat this procedure twice more, each time with a fresh 1.0 mL sample of standard ascorbic acid.
5. In a similar fashion, titrate three blanks, each containing 5.0 mL of 4% Metaphosphoric acid/ acetic acid solution and 1.0 mL of DIW. Average the results for each series of measurements.

B. Unknown ascorbic acid solution:

1. Follow the previous procedure with the supplied unknown sample of Ascorbic acid.

C. Fruit extract:

1. Pour about 15 mL of lime juice into a small beaker and dilute it to 50 mL with the Metaphosphoric acid/ acetic acid solution. Filter it through a rapid flow, fluted filter paper.
2. Pipette 10.0 mL of the filtrate into a 50 mL conical flask and titrate (three times) with DCIP as previously described.
3. Perform blank titration using 10.0 mL of DIW.
4. To test for the presence of interfering agents, pipette 10.0 mL of fresh sample, add few crystal of ascorbic acid oxidase to destroy the ascorbic acid. Let stand, after gentle mixing for 10 minutes followed by dilution to 50 mL by Metaphosphoric acid/ acetic acid solution. Titrate as usual with DCIP solution.
5. Calculate the ascorbic acid concentration in the unknown sample, fruit juice, Vitamin tablets in units of mg/mL.

Expt. No. 4b: Analysis of Leaf Pigments

The most abundant plant pigments are chlorophyll a and b, which occur in a ratio approx. 3:1. The second group of plant pigment, the carotenoids can be divided into two different types; the carotenes and the xanthophylls. Chlorophyll extracted in 80% acetone from a green leaf appears green. The chlorophyll extract can vary in the depth of green or tint of green depending on the plant material from which it was extracted. In Angiosperms (most land plants) there are typically two types of Chlorophyll (Chl) molecules, namely, chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*). Both of these pigments absorb photons of light in the blue and red spectral regions, but the specific wavelengths of light they absorb are different. The absorbance of photons at 663 nm and 645 nm, specific for Chl *a* and Chl *b*, respectively, will be determined. Because the absorption spectra (an integrated picture of the wavelengths of light absorbed) of these two Chl molecules overlap, you will use a simultaneous equation to solve for the amount of both pigments. The equation for this has been worked out and is known as Arnon's equation. Arnon's equation will provide you with quantitative information about the Chl *a* and Chl *b*. In contrast to the chlorophylls, which absorb light in two regions of the visible spectrum, the carotenoids exhibit intense absorption in just one, 350-500 nm.

You will be given different types of leaves from which you will determine the amounts of chlorophyll *a*, b and carotenoids.

Spectrophotometric Determination of Chlorophyll

Procedure:
For the different types of leaves provided follow the procedure given below:

1. Cut the leaves into small pieces. Discard major veins and any tough, fibrous tissue. **Weigh the pieces: you should keep about 0.10 g (100 mg)** of material for grinding (record total fresh weight of each sample).
2. Put the tissue into a mortar and add 10 ml of 80% acetone (acetone:water 80:20 v:v). Grind the tissue with a pestle. You want to pulverize the tissue completely, thus a few grains of sand may help. This is your leaf **homogenate**.
3. Filter the leaf homogenate through the filter paper. The **retentate** is removed by the filter paper (and discarded) and the **extract** (or **filtrate**) is collected in a test-tube.

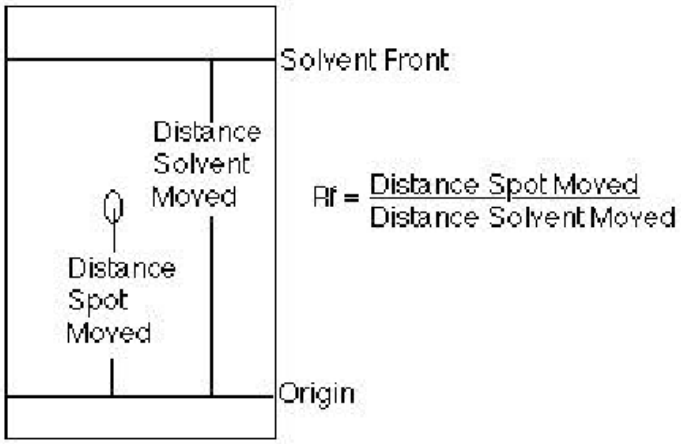
Determination of chlorophyll concentration

1. Obtain a clean cuvette for the spectrophotometer/colorimeter and fill two-thirds with 80% acetone; this is the blank. Wipe the cuvette with a tissue and put it into the spectrophotometer, then set the wavelength to 663 nm. Cover the cuvette chamber and set the spectrophotometer to 0 absorbance with the blank in place. Remove the blank and save for the next measurement.
2. Gently swirl your first extract in the test-tube and fill a second cuvette two-thirds full. Wipe it clean, insert into the spectrophotometer, and close the hatch. The readout should give you the absorbance at 663 nm, the A_{663}. Record this number, and repeat step 2 with the other extracts.
3. Change the wavelength to 645 nm. Reinsert the blank cuvette, and re-zero the spectrophotometer at the new wavelength. Remove the blank and insert a cuvette containing your first extract. Read and record A_{645}. Repeat for the other extracts.

Calculations:
Use Arnon's equation (below) to convert absorbance measurements to mg Chl g^{-1} leaf tissue
Chl $_a$ (mg g^{-1}) = [(12.7 × A_{663}) - (2.6 × A_{645})] × ml acetone / mg leaf tissue
Chl $_b$ (mg g^{-1}) = [(22.9 × A_{645}) - (4.68 × A_{663})] × ml acetone / mg leaf tissue

Total Chl = Chl $_a$ + Chl $_b$
C_{x+c} = [1000 A_{470} – 1.90Chl_a – 63.14 Chl_b]/214, (x = xanthophylls and carotenes)

Thin Layer Chromatography (TLC) of Leaf Pigments
TLC plates coated with silica (stationary phase) will be provided. The mobile phase used is methanol which is more polar than the stationary phase and will migrate from the bottom to the top of the plate. Molecules will separate based on their differential solubility. The more hydrophilic the molecule, the more it is soluble in the methanol and the faster it migrates. The more hydrophobic the molecule, the more time it spends partitioning into the stationary phase and the less distance it migrates with the mobile phase. Migration is measured as a ratio of the distance migrated from the origin, relative to the distance migrated by the solvent front. This ratio is termed the Rf.



Procedure:
Mark the origin with a faint pencil line about 5 mm from the bottom edge of the plate. Add the samples to the origin by lightly touching a small dropper/pipette to the surface of the plate. It is important to dry spots between adding more drops. Each plate should have sufficient room for analysis of two or three samples. Once the plate is loaded and dry, it can be placed in a chromatography chamber (beaker with about 3 mm of methanol in the bottom) in a fume hood. The solvent (methanol) will start to migrate up the plate and pigments will follow. Remove the plate from the chamber when the solvent front is at or very near the top of the plate. Mark the solvent front location with a soft pencil.
Record the Rf colors and fluorescence of the pigments you have fractionated.
Common Plant Pigments in Order of Increasing Polarity:
beta-carotene yellow
chlorophyll *a* blue-green
chlorophyll *b* apple-green
lutein + zeaxanthin yellow
violaxanthin yellow
neoxanthin yellow

Expt. No. 5: Isolation of Genomic DNA from Wheat

Reagents:

- DNA extraction buffer: 25 mM Tris-HCl (pH = 8.0), 50 mM EDTA, 0.5% SDS (Before using, add β-mercaptoethanol to a final concentration of 2%)
- Phenol-chloroform (1:1)
- 3 M sodium acetate (pH 5.2)
- Rnase A
- Proteinase K
- iso-Propanol and ethanol

Protocol:

- Take 4-5 wheat seeds (~100 mg) in mortar containing liquid nitrogen; grind the frozen wheat to a fine powder with a pestle. Transfer powder into 1.5 ml Eppendorf tube (quickly so that it does not become wet).
- Add 500 μL of DNA extraction buffer (containing 10 μL of β–mercaptoethanol) and mix gently by inversion until mixed thoroughly.
- Incubate the tube at 55-60 ^{o}C for 15 min and vortex gently upside down every 2-3 min.
- After the tube cools to room temperature, add 500 μL of phenol-chloroform (1:1) mixture and gently mix until the mixture emulsifies.
- Centrifuge for 10 min, at 5000 rpm at rt.
- Take the supernatant and repeat the phenol-chloroform extraction once more.
- Add 0.1 ml of 3M NaOAc (pH 5.2) and 0.25 ml of ice-cold isopropanol (-15 ^{o}C) to the supernatant and mix gently by inversion. Place the tube at -15 ^{o}C (deep freeze chamber) for 15 min to precipitate the extracted DNA. Centrifuge at 10,000 rpm for 10 min. Decant the supernatant with micropipette.
- Wash the pellet twice in 75% ethanol.
- Resuspend the DNA in 200 μL Tris-EDTA buffer (or Tris-HCl buffer), add 5 μL Rnase A, and incubate at 37 ^{o}C for 20 min.
- Add 500 μL of ice-cold iso-propanol and mix gently; spin at 10,000 rpm for 10 min. at rt. Decant the supernatant with micropipette.
- Resuspend the DNA in 200 μL Tris-EDTA buffer (or Tris-HCl buffer), add 5 μL, and Proteinase K and incubate at 37 ^{o}C for 20 min.
- Add 500 μL of ice-cold iso-propanol and mix gently; spin at 10,000 rpm for 10 min. at rt. Decant the supernatant with micropipette.
- Wash the pellet twice in 75% ethanol.
- Dissolve the DNA pellet in Tris-EDTA buffer (or Tris-HCl buffer) and record the absorbance at 260 nm and 280 nm.

Characterization of DNA:

Nucleic acids absorb ultraviolet radiation in the wavelength range, 200 - 320 nm (i.e. in the UV-B and UV-C region of the electromagnetic spectrum). The absorption maximum in this region is around 260 nm and is due to absorption by the purine and pyrimidine bases. The shape of the spectrum is therefore the same for both RNA and DNA, and they can be detected at concentrations as low as 2.5 μg/ml. **A solution of double-stranded**

DNA with a concentration of 50 μg/ml has an A260 of 1.0. (This value varies slightly depending on the mol% (G+C) of the DNA; but for most purposes the variation can be ignored.) The usual procedure is to measure the A260 of two or three dilutions of your DNA sample to arrive at a reproducible value within the absorbance range, $0.05 \leq A260 \leq 1.5$ (a brief discussion of the linear range of absorbance measurements is given in the attached appendix material). The concentration is then determined by: **C = A260(observed) x 50 μg/ml** (the concentration of the original DNA sample is given by C x dilution factor for the measured sample). UV absorbance can also be used to characterize the purity of a DNA sample. This involves using wavelengths other than 260 nm. Some empirical rules are:

- A320 should be < 0.1A260; values greater than this indicate particulates or other undesirable materials in the preparation of DNA.

- Aromatic amino acids absorb UV light maximally at 280 nm; thus the A260/A280 ratio of a DNA solution is often used as an indicator of protein --- or RNA --- contamination: the ratio for pure, double-stranded DNA should be between 1.65-1.85; higher ratios are often due to RNA contamination, lower ratios can indicate the presence of protein (or phenol, if that is used to purify the DNA).

- Because many proteins do not contain a high amount of aromatic amino acids, a better indicator of protein contamination is the A234/A260 ratio: nucleic acids have an absorption minimum at 234 nm and protein contamination results in an increase in this ratio due to absorption of amino acids (i.e. the ratio should be < 0.5).

- DNA solutions used for absorbance measurements should not have high EDTA concentrations (i.e. > 1 mM), since this compound also absorbs at these wavelengths.

Outline of a DNA extraction:

There are three basic steps in a DNA extraction, the details of which may vary depending on the type of sample and any substances that may interfere with the extraction and subsequent analysis.

- Chelate divalent cations such as Mg^{2+} and Ca^{2+} to stop dnase enzymes functioning and degrading the DNA.

- Break open cells by grinding or sonication, and remove membrane lipids by adding a detergent (SDS).

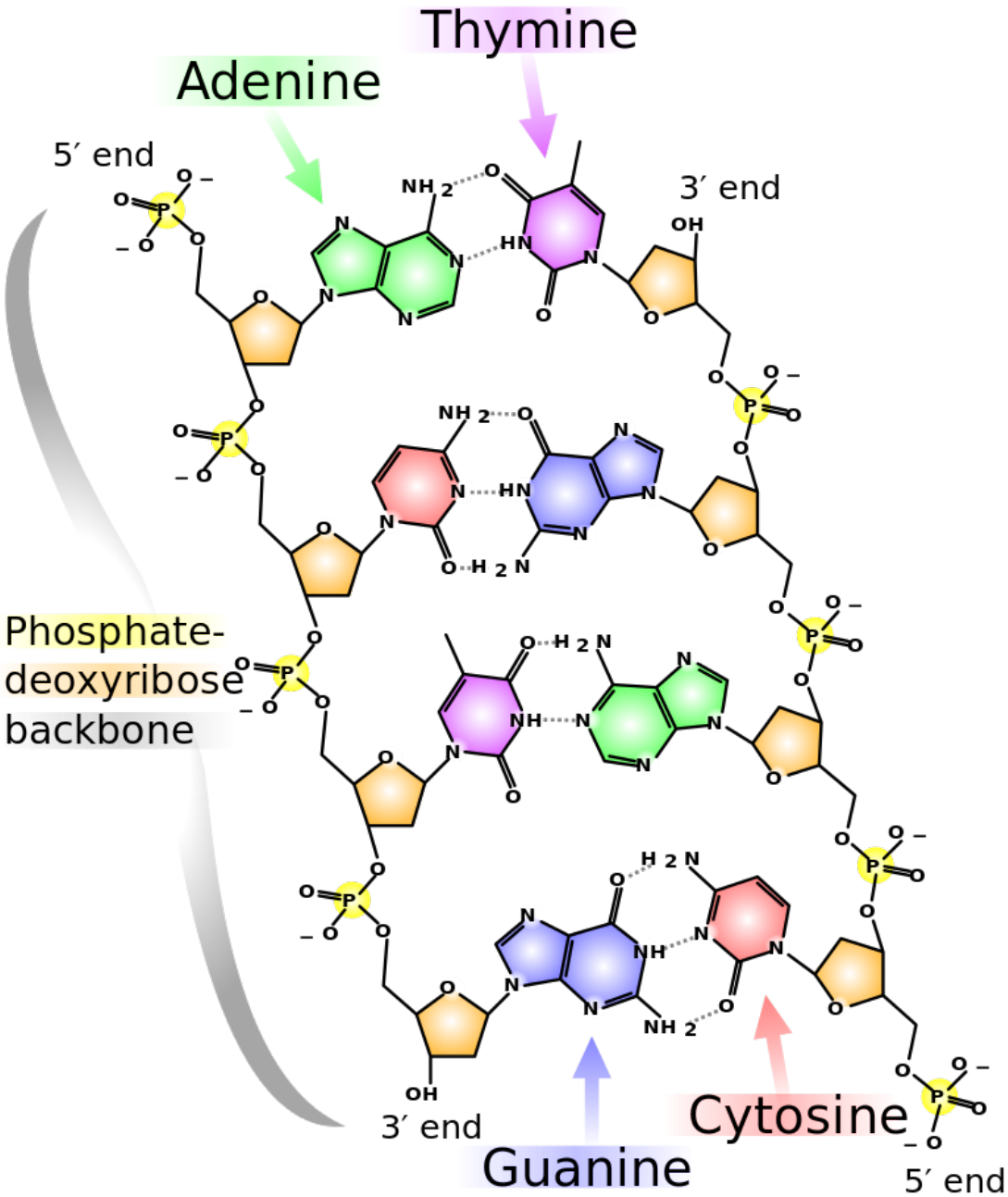
- Remove cellular and histone proteins bound to the DNA, by adding a protease, by precipitation with sodium or ammonium acetate, or by using a phenol-chloroform extraction step.

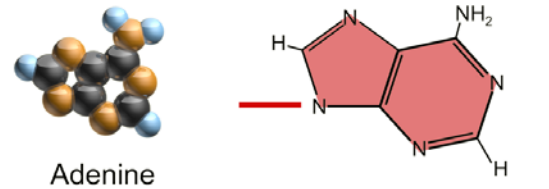
- Precipitate DNA in cold ethanol or isopropanol, DNA is insoluble in alcohol and clings together; this step also removes salt.

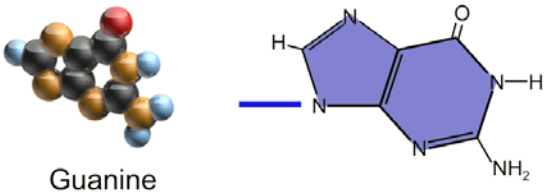
- Wash the resulting DNA pellet with alcohol.

- Solubilize the DNA in a slightly alkaline buffer

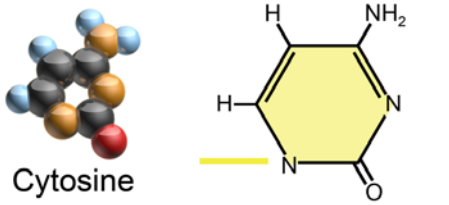
DNA double helix

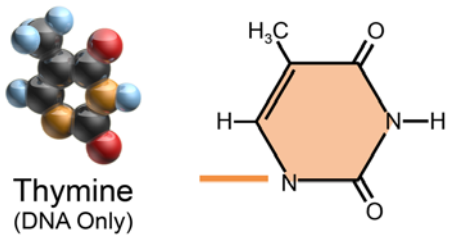


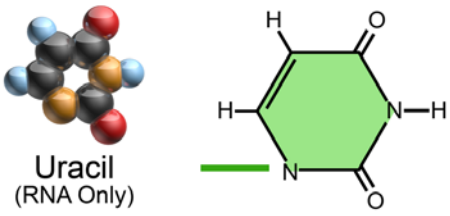




Purines







Pyrimidines