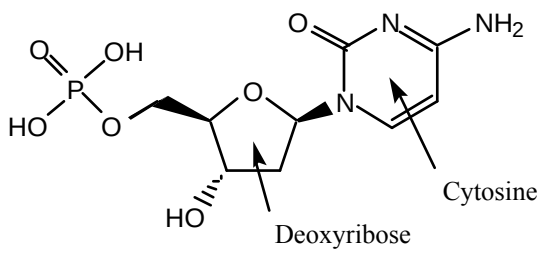
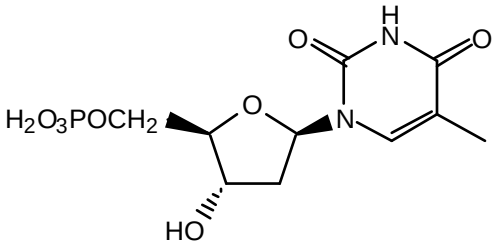
30. DNA Composition by High-Performance Liquid Chromatography[1]

This experiment illustrates quantitative analysis by high performance liquid chromatography (HPLC). It uses only aqueous eluent, so there is no hazardous waste to dispose.

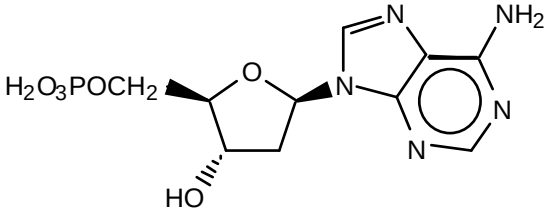
The genetic material deoxyribonucleic acid, is a polymer made of four nucleotides abbreviated C, T, A, and G:



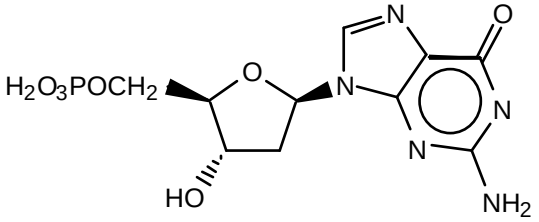
2'-Deoxycytidine 5'-monophosphate (C)



Thymidine 5'-monophosphate (T)



2'-Deoxyadenosine 5'-monophosphate (A)



2'-Deoxyguanosine 5'-monophosphate (G)

In double-stranded DNA, C is hydrogen bonded to G and A is hydrogen bonded to T. Therefore the concentrations of C and G are equal and the concentrations of A and T are equal. DNA from different organisms has different relative amounts of (C + G) and (A + T). When DNA is hydrolyzed by the enzyme nuclease P_1, it is cleanly broken into the four nucleotides.

REAGENTS

Standard nucleotide solution: The standard should contain accurately weighed quantities of the nucleotide monophosphates[2] at concentrations of ~20 mM. The molecular masses of the free acids are C - 307.2, T - 322.2, A - 331.2, G - 347.2. Place the required quantities of the solid acids in a 5-mL volumetric flask and add 2 mL of water and 1.6 mL of 0.10 M NaOH (2 mol NaOH per mol of nucleotide). Dissolve the solid, dilute to the mark with water, mix well, and store the standard in a refrigerator. (The volume of the standard changes when it is cooled, but this is not important. Only the relative concentrations of nucleotides in the standard are important in this experiment.)

Hydrolyzed DNA: The volumes of DNA and nuclease P_1 solutions should be the minimum required for the number of people doing the experiment. Prepare a solution containing 1

mg/mL of calf thymus (or other) DNA.[2] Dissociate the DNA into single strands by heating at 100°C for 10 min and then cooling immediately on ice. Prepare nuclease P_1[2] at a final concentration of 5 units/mL[3] in 50 mM sodium acetate buffer (pH 5.3) containing 0.6 mM $ZnCl_2$. Mix 20 µL of DNA solution with 20 µL of nuclease solution in a small vial with a conical bottom. Heat the vial at 50°C for 1 h and analyze it immediately or store it in the refrigerator.

HPLC Eluent: Prepare 0.010 M potassium phosphate buffer by dissolving 0.010 mol K_2HPO_4 in 800 mL of water, titrating with ~1 M HCl to pH 7.2, and diluting to 1.00 L.

CHROMATOGRAPHY

1. A variety of C_{18}-silica columns should work in this experiment. A 0.46 × 15 cm column with 5 µm particles or a 0.46 × 25 cm column with 10 µm particles are reasonable. Equilibrate the column with 20 empty column volumes of 0.010 M phosphate buffer (pH 7.2) at a flow rate of 1.2 mL/min before beginning chromatography. Establish a flat baseline with an ultraviolet detector at or near 260 nm.

2. Inject 10 µL of the nucleotide standard. You should observe a clean separation of all four peaks (C < T < G < A) with an elution time of 5-10 min. Measure the areas of all four peaks, preferably by computer integration. Alternatively, you can estimate peak area from the formula: area of Gaussian peak = 1.064 × peak height × $w_{1/2,\ \text{where}}$ $w_{1/2}$ is the width at half-height (Figure 23-9 in the textbook). Express the areas of C, T, and A relative to the area of G, which we will define as 1.000. Repeat the procedure with a second injection and measure the relative areas. List the relative peak areas in each run and the average of the two runs.

3. Inject 10 µL of hydrolyzed DNA and measure the relative areas of the peaks. Repeat the process a second time. List the relative areas in each run and the average of the two runs.

CALCULATIONS

1. From the average peak areas of the two standard runs, find the response factors for C, T, and A relative to G. For example, the response factor for C is obtained from the equation

$$\frac{\text{area of C}}{\text{concentration of C}} = F\left(\frac{\text{area of G}}{\text{concentration of G}}\right)$$

$$\frac{A_C}{[C]} = F\left(\frac{A_G}{[G]}\right)$$

There will be similar equations for T, and A. We are using G as the internal standard.

2. From the average peak areas of the two injections of hydrolyzed DNA, find the relative concentrations [C]/[G], [T]/[G], and [A]/[G] by using the response factors from the standard mixture. What is the theoretical value of [C]/[G]? What is the theoretical relationship between [T]/[G] and [A]/[G]?

3. Find the fraction of nucleotides that are C + G by evaluating the expression

$$\textit{Fraction of C + G:} \qquad \frac{[C] + [G]}{[C] + [G] + [A] + [T]} = \frac{\dfrac{[C]}{[G]} + \dfrac{[G]}{[G]}}{\dfrac{[C]}{[G]} + \dfrac{[G]}{[G]} + \dfrac{[A]}{[G]} + \dfrac{[T]}{[G]}}$$

For calf thymus DNA, the literature value of the fraction of C + G is 0.42.

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1. S. M. Wietstock, *J. Chem. Ed.* **1995**, *72*, 950.
2. Sigma, P. O. Box 14508, St. Louis, MO, 63178 Phone: 800-325-3010. www.sigma-aldrich.com
3. For nuclease P_1, one unit is defined as the amount that will liberate 1.0 μmol of acid soluble nucleotides from yeast ribonucleic acid per min at pH 5.3 at 37°C. The commercial preparation has at least 200 units/mg of nuclease P_1.