PRACTICAL SYLLABUS

1. Verification of Chargaff's rule by paper chromatography.
2. Ultraviolet absorption spectrum of DNA and RNA.
3. Determination of DNA and RNA concentration by A_{260nm}.
4. Determination of the melting temperature and GC content of DNA.
5. A study on the viscosity of DNA solutions.
6. Isolation of chromosomal DNA from *E. coli* cells.

EXPERIMENT 1 - Verification of Chargaff's rule by paper chromatography.

Aim: To determine the nucleotides separated by paper chromatography

Procedure:

The nucleotides derived from the hydrolysis of nucleic acids can be separated by paper chromatography.

Mix 100 mg of nucleic acid with 1 ml of 72% perchloric acid and heat on a boiling water bath for 1 hour. Cover the tube with a marble to prevent evaporation [CAREFULLY!]. Cool, add 1 ml water and centrifuge at 3000 rpm for 10 minutes to remove any sediments. The supernatant is used for chromatography.

The solvent system used is:

Isopropanol-water-conc. HCl 130 : : 37: 33

The spots are detected by exposing the dried paper to ultraviolet light, when the spots quench and appear as dark spots.

For quantitation, the individual spots can be cut out, and transferred to tubes each containing 5 ml of 0.1*N* HCl. After overnight elution, the supernatant is decanted off and by using ultraviolet spectroscopy and the data in the following table, the concentrations are calculated.

Table B 5.1 Absorption Characteristics of Bases

Base	Abs. max	Millimolar extinction coefficient
Adenine	260 nm	13.3
Guanine	250	11.2
Uracil	260	7.9
Thymine	265	7.9
Cytosine	273	10.5

From this calculate the base composition of the DNA or RNA in terms of moles per mg nucleic acid.

EXPERIMENT 2 - Ultraviolet absorption spectrum of DNA and RNA.

Aim: To determine the absorption spectrum of DNA and RNA.

Procedure:

(*a*) Prepare solutions of RNA and DNA in saline-citrate solutions, and take their absorption spectrum in the ultraviolet region. Nucleic acids show strong absorption at 260 nm. Express results as $O.D._{260}$ units/ml solution.

(*b*) Using the same solutions as above, hydrolyse a known sample (Expt. B 5.6) and measure the $O.D._{260}$ of the hydrolysed sample. Calculate the $O.D._{260}$ units per mg RNA and DNA hydrolysed. What differences do you notice?

It will be seen particularly in the case of DNA, that O.D. at 260 nm would show an increase in the hydrolysed sample. This phenomenon is referred to as hyperchromicity. In the intact DNA, the nucleotide bases which are responsible for the absorption at 260 nm are in the hydrogen bonded state in the double strands. In this state their absorption is low. But hydrolysis liberates the bases, and the free bases have greater absorption.

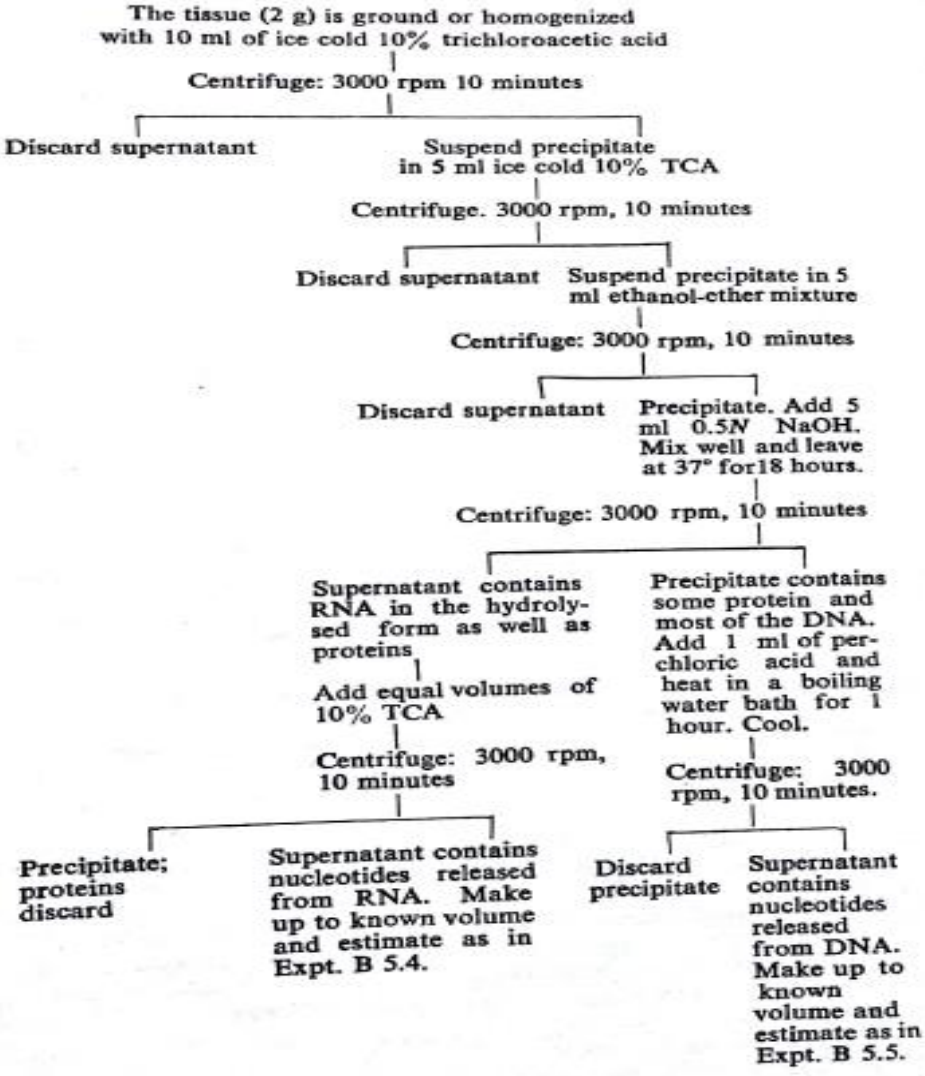
Result:

EXPERIMENT 3 - Determination of DNA and RNA concentration by A260nm.

Aim: To determine the absorption spectrum of DNA and RNA at O.D 260nm.

Procedure:

The application of the above techniques directly to biological samples suffers from the disadvantage of having too many interfering materials since the reagents estimate only the sugar moieties. Therefore, the following (somewhat lengthy) procedure given as a flow chart is used.



EXPERIMENT 4 - Determination of the melting temperature and GC content of DNA.

Aim: To determine the melting temperature and GC content of DNA.

Procedure:

Hyperchromicity as a function of temperature can be demonstrated by heating DNA solution and measuring the O.D. at different temperature. A spectrophotometer, with facilities to heat the cuvettes, would be needed. The following procedure will demonstrate the principle but with less accuracy.

Prepare a DNA solution (use the isolated one or calf thymus DNA) in SSC at a concentration to give an $O.D._{260}$ of 0.1. Have a boiling water bath ready. Arrange a shallow beaker near the spectrophotometer, fill it to half its capacity with water and fix a thermometer. A magnetic stirrer could be fixed if available. Take the DNA solution in a cuvette, keep it immersed in water for 2 min and quickly measure its O.D. at 260 nm. Add a few ml of boiling water to the beaker, mix and note the temperature. Dip the cuvettes for 2 minutes and again measure the O.D. Take as many readings as possible at different temperatures. With a little practice, the operations can be done fast so that the DNA solution in the cuvette is not allowed to cool.

After reaching the temperature of boiling water repeat the above procedures but while cooling by adding cold water.

Analysis of Data

If the DNA sample used is double stranded and not denatured, a sharp increase in $O.D._{260}$ will be observed between 60-80°C. The midpoint of this increasing slope is called T_m value or melting temperature of DNA.

Due to heating, the hydrogen bonds, connecting the two strands of the DNA are split and this causes the O.D. to increase. The temperature at which the two strands separate depends on the base composition and is characteristic of a DNA species.

The cooling curve will show reassociation of the two strands. This is called annealing. This technique of melting of strands and their reassociation is being used extensively in the isolation of genes and their characterization, etc.

Expt. B 5.10 Correlation of T_m and Base Composition

Measure the T_m value of DNA samples obtained from different sources. Using the same solutions, determine the base compositions as done in Expt. B 5.7 and express the values as mole percent. Draw a graph with $(G + C)$ values against T_m and see whether there is any correlation.

EXPERIMENT 5 - A study on the viscosity of DNA solutions

Aim: To determine the viscosity of DNA solutions.

Weigh out sufficient amount of DNA and suspend in 7.5 ml of 0.1*M* acetate buffer (pH 4.5) to make a 0.2% solution. Dissolve the DNA carefully by gentle stirring with glass rod to obtain a viscous solution. Transfer to a test tube. In another tube take acetate buffer. Using a 0.1 ml pipette, draw the acetate buffer solution to the zero mark. Keep the pipette end touching the solution. Now open the closed end, simultaneously starting the stopwatch. Stop the watch as soon as the level drops to a fixed point in the pipette, say 0.05 ml. Repeat a few times and obtain the average. Repeat the same procedure with the DNA solution, preferably using the same pipette. Calculate the average time.

The relative viscosity of the DNA samples is roughly given by the time taken for the DNA solution divided by the time taken for the buffer to fall through the same distance.

Distribute the DNA solution into several test tubes, about 0.5 ml in each. Then heat a water bath *slowly* and keep track of the temperature. At intervals of about 10°, keep one tube of DNA in the water bath for exactly two minutes and immediately transfer it to ice. Repeat till about the water boiling temperature. Measure the relative viscosity of the DNA samples kept in ice.

Draw a graph with temperature and relative viscosity and comment on results.

EXPERIMENT 6 - Isolation of chromosomal DNA from *E. coli* cells.

Aim: To isolate total genomic DNA from the bacterial cells and visualizing the same DNA by gel electrophoresis.

Principle:

Genomic DNA preparation differs from the plasmid DNA preparation. Genomic DNA is extracted from bacterial cells by immediate and complete lysis whereas plasmid DNA is isolated by slow-cell lysis to form a sphaeroplast.

The procedure of genomic DNA extraction can be divided into 4 stages:

1. A culture of bacterial cell is grown and harvested.
2. The cells are broken open to release their contents.
3. The cells extracted are treated to remove all components except the DNA.
4. The resulting DNA is then purified.

Materials required:

1. TE buffer (pH 8.0)
2. 10% (w/v) sodium dodecyl sulfate (SDS)
3. 20 mg/mL proteinase K
4. Phenol/chloroform (50:50)
5. Isopropanol
6. 70% ethanol
7. 3M sodium acetate pH 5.2

Procedure:

1) *E. coli* culture was grown overnight in Luria broth.
2) The overnight culture was transfered into 1.5 mL to a microcentrifuge tube and centrifuged at 10000 rpm for 5 min. The supernatant was decanted and repeated with another 1.5 mL of cells.
3) The bacterial pellet was resuspended in 467 µL TE buffer by repeated pipetting. 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K was added, mixed, and incubated for 1 hr at 37°C.

4) Equal volume of buffer saturated phenol: chloroform: isoamyl alcohol (25:24:1) or chloroform: Isoamyl alcohol (24:1) was added and mixed well by inverting the tube until the phases are completely mixed. (Caution: Phenol causes severe burns. Wear gloves, goggles, and a lab coat, and keep tubes capped tightly)
5) Centrifuged at 12000 rpm for 5 min.
6) The upper aqueous layer phase was transferred to a new 1.5 ml eppendorf tube and equal volume of chloroform:Isoamyl alcohol (24:1) was added, Again mixed well by inverting and transfered to a new tube and centrifuged at 12000 rpm for 5 minutes.
7) The upper aqueous phase was again transferred to a new 1.5 ml eppendorf tube.
8) To the aqueous phase, 1/10 volume of 3M sodium acetate (pH 5.2) was added.
9) Then 0.6 volume of isopropanol was added and mixed gently until the DNA gets precipitates.
10) Centrifuged at 12000 rpm for 10 min and the supernatant was discarded.
11) The DNA pellet was washed by adding 200 µL of 70% ethanol (Centrifuge at 10000 rpm for 3 min)
12) Finally the DNA pellet was suspended in a 100–200 µL TE buffer. Complete resuspension may take several days.
13) After DNA has dissolved, the purity of the DNA was checked by electrophoresis and spectrophotometric analysis.
14) 0.8 % agarose gel was prepared using 1X TAE buffer.
15) Isolated DNA samples were loaded into the wells, recording which samples are loaded into which wells as lane 1, 2, etc. The power supply was started after sample loading, with the voltage set to 50 V.
16) Ran the gel until the second dye from the well has reached 3/4 th of the gel.
17) After the run was completed, the gel was observed under UV transilluminater.
18) Then DNA was stored at 4°C short term, –20°C or –80°C long term

Results and Observations: