ADARSH COLLEGE

OMERGA, DIST. OSMANABAD

DEPARTMENT OF MICROBIOLOGY

CERTIFICATE

Practical paper – III ***Bioinstrumentation Techniques and Applications***

Certified that Shri / Miss_____

has satisfactorily completed the course of practical work in M. Sc. I YEAR (SEMESTER I) prescribed by Dr. B. A. M. University, Aurangabad under my supervision in the Microbiology Laboratory during the academic year 20 – 20

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Head of the department

Date:

Signature of the Examiner

Date:

Exam Seat No. _____

Paper –III **Bioinstrumentation Techniques and Applications**

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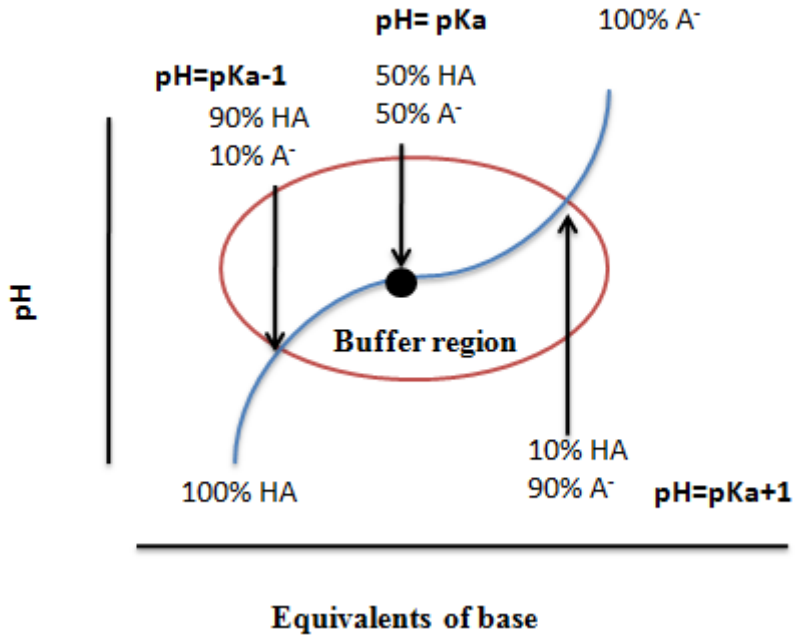
Expt. No. 1 Date

Studies on pH titration curves of amino acids and determination of pKa values and Handerson-Hasselbach equation.

Aim: To determine the titration curve for an amino acid and to use this curve to estimate the pKa values of the ionizable groups of the amino acid.

Theory:

Titration curves are obtained when the pH of given volume of a sample solution varies after successive addition of acid or alkali. The curves are usually plots of pH against the volume of titrant added or more correctly against the number of equivalents added per mole of the sample. This curve empirically defines several characteristics. The precise number of each characteristic depends on the nature of the acid being titrated: 1) the number of ionizing groups, 2) the pKa of the ionizing group(s), 3) the buffer region(s).



Amino Acids are weak Polyprotic Acids. They are present as zwitter ions at neutral pH and are amphoteric molecules that can be titrated with both acid and alkali. All of the amino acids have an acidic group (COOH) and a basic group (NH_2) attached to the α carbon, and also they contain ionizable groups that act as weak acids or bases, giving off or taking on protons when the pH is altered.

The strong positive charge on the amino group induces a tendency for the carboxylic acid group to lose a proton, so amino acids are considered to be strong acids. Some amino acids have other ionizable groups in their side chains and these can also be titrated.

When an amino acid is dissolved in water it exists predominantly in the isoelectric form. The isoelectric point, pI, is the pH of an aqueous solution of an amino acid at which the molecules have no net charge. In other words, the positively charged groups are exactly balanced by the negatively charged groups. When this dissolved amino acid is titrated with acid, it acts as a base, and with base, it acts as an acid which makes them an amphoteric molecule.

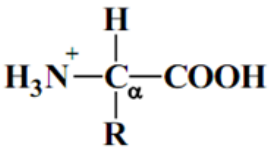
These ionizations follow the Henderson-Hasselbalch equation:

$$pH = pKa + log\frac{[unprotonated\ form\ (base)]}{[Protonated\ form\ (acid)]}$$

When the concentration of the unprotonated form equals that of the unprotonated form, the ratio of their concentrations equals 1, and log 1=0. Hence, pKa can be defined as the pH at which the concentrations of the protonated and unprotonated forms of a particular ionizable species are equal. The pKa also equals the pH at which the ionizable group is at its best buffering capacity; that is the pH at which the solution resists changes in pH most effectively.

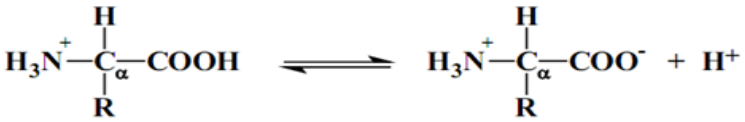
The pK is the pH at the midpoint of the buffering region (where the pH changes only slightly upon addition of either acid or base). The pK is the pH corresponding to the inflection point in the titration curve. The end point of a titration curve represents the observed end of the titration. The isoelectric point (isoelectric pH; pI) is the pH at which the amino acid has a net zero charge. For a simple diprotic amino acid, the pI falls halfway between the two pK values. For acidic amino acids, the pI is given by ½(pK1 + pK2) and for basic amino acids it's given by ½(pK2 + pK3).

In this experiment we are finding out the titration curve of the amino acid Glycine.

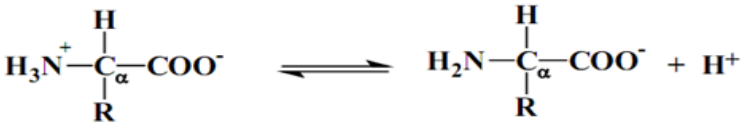


Glycine is a diprotic amino acid which means that it has two dissociable Protons, one on the α-amino group and the other on the carboxyl group. In the case of Glycine, the R group does not contribute a dissociable Proton.

Dissociation 1:



Dissociation 2:



The dissociation of proton proceeds in a certain order which depends on the acidity of the proton: the one which is most acidic and having a lower pKa will dissociate first. So, the H+ on the α-COOH group (pKa1) will dissociate before that on the α-NH3 group (pKa2).

Materials Required:

1. 0.1M Hydrochloric acid
2. 0.1M Sodium Hydroxide
3. pH Meter
4. 0.1M Glycine (7.5 gm in 100 ml water)
5. Burette -2
6. Beaker
7. Stirrer
8. Standard Buffer of pH=4, pH= 7, pH=10

pH 4

Sol A: 0.2 M solution of acetic acid (1.15 ml in 100 ml)

Sol B: 0.2 M solution of Sodium acetate (1.64 gm in 100 ml)

41 ml of A + 9 ml of B diluted to total 100 ml.

pH 7

Sol A: 0.2 M solution of monobasic sodium phosphate (3.12 gm in 100 ml)

Sol B: 0.2 M solution of dibasic sodium phosphate (2.83 g of Na_2HPO_4 or 7.17 g of $Na_2HPO_4.12\ H_2O$ in 100 ml)

19.5 ml of sol A + 30.5 ml of sol B diluted to total 100 ml.

pH 10

Sol A: 0.2 M solution of Sodium Carbonate (2.12 gm in 100 ml)

Sol B: 0.2 M solution of Sodium Bicarbonate (1.68 gm in 100 ml)

14 ml of sol A + 11 ml of sol B diluted to total 100 ml.

Materials Required:

1. 0.1M Hydrochloric acid
2. 0.1M Sodium Hydroxide
3. pH Meter
4. 0.1M Glycine
5. Burette -2
6. Beaker
7. Stirrer
8. Standard Buffer of pH=4, pH= 7, pH=10

Procedure:

1. Pipette out 20ml of the amino acid solution into a 100ml beaker.
2. Standardize the pH meter using the standard buffer solutions.
3. Determine the pH of the amino acid solution.
4. Add 0.3ml of 0.1M HCl from the burette and record the pH after each addition.
5. Continue adding the acid until the pH falls to 1.6
6. Wash thoroughly the pH electrode in distilled water.
7. Take 20 ml of amino acid solution in another beaker and check its pH.
8. Now titrate the amino acid solution by adding 0.3ml of 0.1M NaOH until the pH reaches 12.5.
9. Plot the titration curve using the values recorded and find the pKa values.

Tabular Column:

Volume of NaOH added (ml)	pH = 6.22

Volume of HCl added (ml)	pH = 6.22

Expt No. 2 **Date**

Separation of amino acids by Paper Chromatography

Aim: - To separate amino acids in a given mixture by paper chromatography.

Theory / Approach: -

Chromatography is an analytical or separatory procedure, which permits the separation of one or more biological compounds from a mixture. This technique was employed by M. Tswet to separate coloured pigments. It is highly efficient laboratory technique. It consists of two phases. One is stationary phase and other is mobile phase. The choice of stationary or mobile phase is made so that the compounds to be separated have different distributory coefficients.

The various chromatographic techniques based upon the choice of stationary and mobile phases are –

1. Column Adsorption chromatography
2. Thin layer chromatography
3. Paper chromatography
4. Gas liquid chromatography
5. Ion exchange chromatography

Paper chromatography: -

In paper chromatography, paper absorbs water from the atmosphere. This water is held between cellulose fibres of the paper and is regarded as one of the solvents. The mobile phase (a non aqueous solvent) is allowed to travel along the paper by capillary action. The solute molecules on the paper distribute themselves between the two phases in a ratio, which is characteristic of their distribution coefficients. The more soluble a solute is in the mobile phase, the further will this compound travel along the paper in the direction of flow of solvent. The distance moved by the solute in the direction of solvent flow during a chromatographic separation is characterised by the term $\mathbf{R_f}$ value (Relative to front), which is defined as

$$\mathbf{R_f}\text{ value} = \frac{\text{The distance travelled by the solute}}{\text{The distance travelled by the solvent}}$$

Amino acid	Rf value
alanine	0.38
arginine	0.20
asparagine	0.5
aspartic acid	0.24
cysteine	0.4
glutamine	0.13
glutamic acid	0.30
glycine	0.26
histidine	0.11
isoleucine	0.72
leucine	0.73
lysine	0.14
methionine	0.55
phenylalanine	0.68
proline	0.43
serine	0.27
threonine	0.35
tryptophan	0.66
tyrosine	0.45
valine	0.61

Requirements: -

1. Mixture of amino acids
2. Solvent system (n–butanol, Acetic acid and water in a proportion 4 : 1 : 5)
3. Locating reagent Ninhydrin
4. Whatmann filter paper no. 1
5. Chromatographic jar

Procedure: -

1. Cut a piece of Whatmann filter paper no. 1 into strips. Draw a line with pencil across the width of the paper 2.5 cm from the bottom of the strip.
2. At the centre of this line apply the amino acid mixture 2 to 3 times with the help of capillary tube or thread and allow to dry.
3. Put the paper strip into the chromatographic jar containing solvent system so that the bottom of the strip should dip into the solvent 1 cm. A care should be taken that the line of application of mixture should not dip

into the solvent system. The strip should not touch to the sides of the chamber.

4. Close the jar by lid and allow the solvent system to flow about 10 to 15 cm along the strip.
5. Remove the strip, air dry and mark solvent front with pencil.
6. Spray ninhydrin reagent and dry in oven at 60 ^{0} C for 5 to 10 minutes.
7. Observe for colour spots and mark the circle around the spots with pencil and find out the centre of the spots.
8. Note down the distance travelled by the spots from the origin and calculate the $\mathbf{R_f}$ value.
9. Compare the $\mathbf{R_f}$ values with standard $\mathbf{R_f}$ values and identify the amino acids.

Observation: -

The distance travelled by the solvent =

The distance travelled by the spot 1

The distance travelled by the spot 2

Calculations: -

$\mathbf{R_f}$ value of spot 1 = _____

$\mathbf{R_f}$ value of spot 2 = _____

Result : -

In the given mixture the amino acids are –

1.

2.

Conclusion: - By the paper chromatography method amino acids can be separated from the mixture and can be identified by comparing the observed $\mathbf{R_f}$ values with standard $\mathbf{R_f}$ values.

Expt. No. 03 **Date**

Separation of serum proteins using Agarose Gel Electrophoresis

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Aim: - To separate serum proteins by agarose gel electrophoresis

Theory / Approach: -

Electrophoresis is the term used to describe the movement of charged ions in the solutions under the influence of an electric field. The positive charged particles move towards cathode and negatively charged particles towards anode.

As a result of different rates of migration, a complex mixture can be separated into a number of fractions of different ionic mobility. The greatest usefulness of electrophoresis is in clinical diagnosis for serum, urine, spinal fluids, gastric juice and other body fluids.

The electrophoresis is of following types---

1. Paper electrophoresis
2. Cellulose acetate electrophoresis
3. Starch gel electrophoresis
4. Agar gel electrophoresis
5. Polyacrylamide gel electrophoresis (PAGE)

The pH of the solution plays major role in determining the mobilities of ions, since the net charge carried by most ions is pH dependent. This is particularly important where Zwitter ionic species such as amino acids or proteins are concerned.

According to the position of separating media it is either vertical electrophoresis or horizontal electrophoresis. Great varieties of buffer such as Barbiturate, Phosphate, Acetate, Citrate, EDTA etc, which cover the pH range 1 to 11 are used in the electrophoresis.

The isoelectric point can be defined, as a pH at which there is minimum concentration of an electrically focused ampholite. In alkaline buffer, serum protein migrates towards positive pole. Albumin migrates in advance of other fractions with α_1, α_2, β, and γ globulin in succession.

Requirements: -

1. Normal serum
2. Borate buffer of pH 8.6

Composition: -

A) 0.2 M solution of boric acid (12.4 gms of Boric acid was dissolved in 1 litre of D/W)

B) 0.05 M solution of Borate (19.05 gm of Borate was dissolved in 1 litre of D/W)

C) Complete solution: - 250 ml of A + 87.5 ml of B was added and diluted it to 1000 ml with D/W. The pH of this buffer was adjusted to 8.6

3. Agarose gel 1 % W/V

Preparation: - 1 gm of agarose was dissolved in 100 ml of Borate buffer (pH 8.6). The mixture was boiled to get uniform suspension. place the combs of electrophoresis set such that it is approximately 2 cm away from the cathode.

Pour the agarose solution in the central part of the tank when the temperature reaches approximately 60 ^{0} C. Do not generate air bubbles. The thickness of the gel should be around 0.5 to 0.9 cm. Keep the gel undisturbed at room temperature for the agarose to solidify.

Pour Borate buffer into the gel tank till the buffer level stands at 0.5 to 0.8 cm above the gel surface.

Gently lift the combs, ensure that wells remain intact.

4. Staining reagent (Ponceau S stain 0.2 gm in 100 ml of 5% Trichloro Acetic acid)
5. Loading dye (Glycerol 0.5 ml, Bromophenol Blue 0.5 ml, Serum sample 1 ml)
6. Equipment: - The horizontal electrophoretic chamber

Procedure: - Electrophoresis

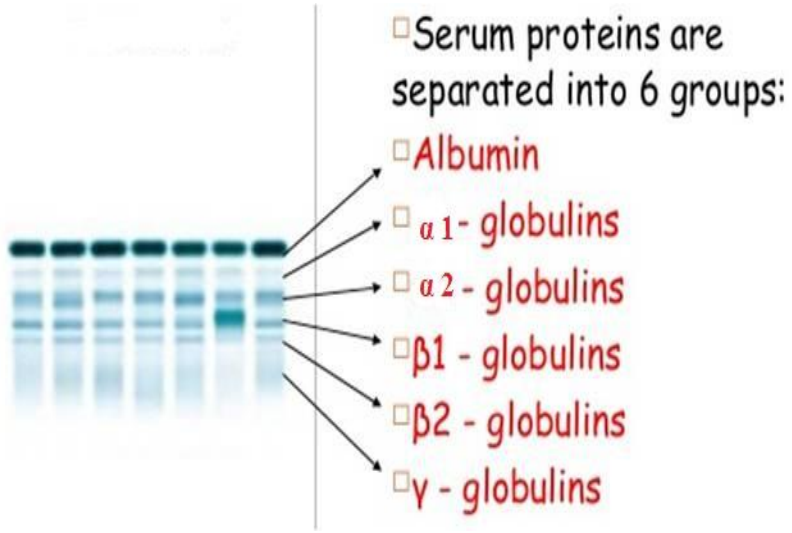
1. Connect the power cord to the electrophoretic power supply according to the convention.
2. Red : Anode. Black : Cathode
3. Load the loading dye in one well and samples in the remaining wells.
4. Set the voltage to 100 volts and the switch on the power supply.
5. Switch off the power when the loading dye from the well reaches 3 / 4 th of the gel. This takes approximately 1 to 2 hours.
6. Staining procedure to visualize protein: -

A) Carefully transfer the gel (from gel tank) into a tray containing Ponceau S stain. Make sure that gel is completely immersed.

B) For uniform staining, keep stain approximately 10 minutes. Shake intermittently every 2 to 3 minutes.

C) Pour out the staining dye into a container. Destain the gel by washing with tap water several times till the protein is visible as a dark band against a light background.

Observation: - Dark bands of proteins are observed.



Result: - Proteins from the serum sample are separated.

Conclusion: - By this method we can separate and observe the proteins from serum sample.

Expt. No. 04 **Date**

Study of UV absorption spectra of different macromolecules

Aim: To determine λ max for different macromolecules

Theory:

The molecules having molecular weight 100 daltons or more are called macromolecules. Macromolecules are of 4 categories- Proteins, Nucleic acids, Polysaccharides and lipids.

Absorption spectrum is a unit pattern of absorption maximum at different wavelength. It is often used in biochemistry to characterize and identify compounds. The energy equivalent for excitation of electrons lies in the ultra violet spectrum hence the absorption spectra are studied by using various wavelengths of UV.

The sample is exposed to light of particular wavelength, the electrons absorb energy in appropriate amount to move to higher orbital and this is reflected in the absorption of certain wavelength. Analysis of transmitted light after suitable splitting to separate the wavelength reveals the nature of absorption of the sample. The spectrum of the compound in the visible or UV region is typical characteristic of the molecule. The absorption peaks are usually referred as λ max for any compound.

The extent of absorption of one molecule is maximum when it is in pure state. Thus two applications of spectral studies are

1. To check purity of compound
2. To measure concentration of substrate

Nucleic acid: They show maximum absorption in the region of 260nm. The extent of absorption of nucleic acid is a measure of their integrity. Partially degraded nucleic acid strongly absorb in their UV region to conjugate double bonds of purines and pyrimidines.

Proteins: Proteins absorb strongly at 280 nm according to the amino acid tyrosine and trypophan. This provides sensitive and nondestructive forms of assay.

Proteins also absorb lights in UV range because of peptide bonds. Protein solutions absorb in the range of 275 nm due to indole group of tryptophan, phenolic group of tyrosine and too much lesser extent phenolic group of phenylalanine. In practical it is more convenient to measure the extinction at 210 nm where the specific extinction coefficient is about 200 for most proteins. All proteins have a similar specific absorption; since peptide bond content is similar .Pure protein have a ratio of absorption of (at 260nm and 280nm) about 1.8 while nucleic acids have a ratio 0.5.

Requirements:

1. Pure protein solution (Bovine albumin) – 1mg/ml, 2mg/ml, 3mg/ml in water
2. Standard DNA solution- 50 µg / ml, 100µg / ml in saline citrate buffer
   Composition of buffer
   D/W 1000ml
   NaCl 1.5 M (Na 23+ Cl 35.5= 58.5 gm for 1 M) 87.75 gm
   Triodium citrate 0.15 M (258 gm for 1M) 38.7 gm
3. Spectrophotometer
4. Graph paper

Procedure:

1. The above mentioned concentration of protein and DNA are prepared. The absorption spectra taken over in the range of 200 to 300 nm at an interval of 10 nm.
2. With DNA solution saline citrate buffer is used as Blank and for protein D/W is used as Blank.
3. A graph is plotted with wavelength on X axis and Optical Density on Y axis.
4. Optical density at 260 nm and at 280 nm is compared to confirm the presence of DNA. (The ratio of OD at 260 nm and at 280 nm is between 1.5 to 1.6)

Observation table

1. **Absorption spectrum of proteins**

Wavelength in nm	**OD at different conc of protein**		
	1mg/ml	**2mg/ml**	**3mg/ml**
200			
210			
220			
230			
240			
250			
260			
270			
280			
290			
300			

2. Absorption spectrum of DNA

Wavelength in nm	OD at different conc of DNA	
	50µg/ml	100µg/ml
200		
210		
220		
230		
240		
250		
260		
270		
280		
290		
300		

Result:

Absorption spectra for protein and DNA are obtained as per graph. The absorption peak (λ max) for protein solution is at 280 nm and for DNA solution is at 260 nm.

The ratio of OD at 260 nm and at 280 nm is _____.

Conclusion:

As protein sample shows maximum absorption at 280 nm, the presence of protein in given sample is confirmed.

The given DNA sample shows maximum absorption at 260 nm and the ratio of OD at 260 nm and at 280 nm is between 1.5 to 1.6. Hence the presence of DNA in a given sample is confirmed.