⇒ Theory of electrophoresis

Depending on charge they carry, ionised solute move towards cathode / anode in electrophoresis system

ex → Positive ion → migrate to cathode.
Neg. ion → Anode.

- Amphoteric (protein, NA) – having either positive or negative charge.
  ↓
  depends upon pH of solution
  ↙ ↘
  Acidic solut^n → pH less than its pI → becomes +ve charged → migrate towards cathod
  Alkaline solu^n → pH more than its pI → becomes -ve charge → Anode.

→ Rate of migration:-

driving force $F = xQ = \frac{(EMF)Q}{d}$

where F = force exerted on ion.
x = current
Q = net charge
EMF = Electro motive force (voltage)
d = length of electrophoretic medium

→ steady acceleration of migrating ion is counter acted by resisting force;

$$F' = 6\pi r \eta v$$

where F' = resisting force
r = ionic radius of solute
η = viscosity of medium.
v = rate of migration = length travelled / sec.

$$F = F'$$

∴ resultant of 2 forces is constant velocity.

$$\therefore 6\pi r \eta v = xq.$$

$$\therefore \frac{v}{x} = \frac{q}{6\pi r \eta} = [u]$$

→ u is defined as electrophoretic mobility.
↳ rate of migration / unit field strength

directly
u α net charge.
inversely α to size of molecule and viscosity of medium.

⇒ Factors affecting rate of motility
① Net charge of molecule
② Size and shape of molecule
③ Strength of electric field
④ Support media properties
⑤ Ionic strength of buffer
⑥ Temp.
⑦ end osmosis

* Electroendosmosis :-

electrophoresis support medium such as gel

↓

In contact ī water, takes negatively charg

caused by adsorptⁿ of hydroxyl ion.

↓

Fixed to surface and immobile

↓

Positively ions in solution

* Wick flow - movement of buffer in the support medium

Passage of current throug resistive medium

↓

Heat generatⁿ

↓

evaporatⁿ of solvent from medium. (dry

↓

Draws buffer into medium

↓

Affect protein migration

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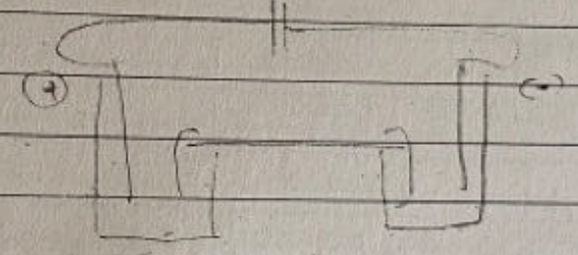
Instrumentation.

① 2 reservoirs → Contain buffer solution
② carbon / platinum electrodes → Supply current from power supply
③ Support medium → where separation takes place.
④ Wicks. → connect medium & buffers or directly to electrode, completing circuit.

↓

entire apparatus is enclosed to prevent evaporation



(1) Power Supply:
Gives non current
Direct power supply (DC)

↓

set the polarity of electrode and delivers current into medium.

↓

Gives movement of ionic species in medium.

- Current flowing through medium has resistance & produces heat

Heat = EIt

E = EMF (V)
I = current (A)
t = time (s)

If constant voltage power supply is used,

As heat released in wet medium

↓

↑ thermal agitation of dissolved ions. and thus ↑ conductance (↑ current)

↓

↑ protein migration and water evaporation.

↓

further ↑ in current.

To minimise these effect, constant-current power supply should be used. → keeping the current constant → ↓ heat effect → stabilize migration rate.

Buffers.

purpose → ① They carry the applied current

② They set pH at ⊆ electrophoresis is carried out

↓

Thus they determine

① Type of electric charge and extent of ionization of solute

② electrode toward ⊆ solute will migrate

→ Ionic strength of buffer determines

① thickness of ionic cloud surrounding charged

② rate of migration and sharpness of band.

↑ Ionic concen → ↑ ionic clouds

↓

hindering movement of molecule

for protein Separat? → Tris. boric EDTA and barbital buffers are used.

Ionic strength of buffer determines.

↓

Ionic cloud surrounding charged molecule

↓

↑ cone. → ↑ size of ionic cloud

↓

hindering of movement of molecule

Current and Heat produc?

$H \propto R I^2$

↑ concentra? of ions → ↓ Resistan → ↑ current

↑ current & heat generat?

Sharp bands & good resolution

excessive heat denature protein and other components.

⇒ to control excessive heating.

① "Submarine" techniques. → temp 10-14°C

↓

② gel submersed in buffer & is cooled by external cooling devices

OR

③ electrophoretic chamber cooled by circulating water / Integral pelties plate seated

OR

④ Compartment of cooled ethylen glycol & is in contact & gel during run.

③ Support media :

purpose → provide matrix in ⊆ protein separation takes place.

Various types → ranges from pure buffer soln (in capillary) to insoluble gel or membrane of cellulose acetate

Separation → based on differences in charge-to-mass ratio of protein and porous size of medium

① Starch → 1st gel medium used
- only historical interest.
- separate protein by charge-to-mass ratio and size
- bez protein are compared on gel surface before migration → form narrow band & good resolution

② Cellulose acetate :
- Acetate ester of cellulose.

Dry membrane → contain 80% of air spaces (in opaque) interlocking cellulose acetate fibres

↓

soaked in buffer

↓

air space filled ⊆ liquid, becomes pliable

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Disadvantage :-

① opacity of stained memb need to be cleared before densitometry
↓
Soaking in 95:5 methanol : glacial acetic acid

② Necessity of presoaking of dried memb
↓
time consuming
↓
largely replaced by Agarose gel.

③ Agarose

- linear polymer of alternating D-galactose and 3,6 anhydro-D galactose
- Obtained from agar by separating from agaropectin → highly charged, containing acidic sulfate and COOH group.

advantage

① larger pore size → separation only depends on charge to mass ratio.

② lower affinity for protein → good migration

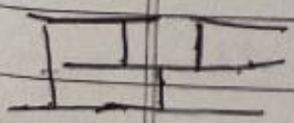
③ No opacity after drying → permits excellent densitometry

④ free of ionizable group → little endosmosis.

→ Procedure of gel formation
1% agarose gel is made by dissolving 100 mg of agarose powder in 10 ml buffer
↓
Heating till clear liquid appear
↓
poured on glass surface and allowed to become gel.
↓
Gel - Samples are applied using comb and allowed to diffuse in gel x 2 mins.
↓
Comb is removed and gel is ready

→ Commercially produced prepackaged microbore gel is also available.

(3) Polyacrylamide gel:-
- polymeric matrix consisting linear chain of acrylamide cross-linked c bis-acrylamide
- Thermostable, transparent and strong.
- Depending on conc., can be made in wide range of pore size eg. 7.5% gel → small pore size



↓
Most protein migrate unimpeded exception are fibrinogen, β lipoprotein α2 microglobulin γ globulin.
↓
molecular radius exceeds

Advantage

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① ~~Thus~~ Thus separation is based on charge - to - mass ratio and size of molecule called molecular sieving

↓

protein can be resolved in to more individual fraction

② gel is uncharge → No endosmosis

Disadvantage

① acrylamide → potential carcinogenic

↓

appropriate cautions must be taken while handling.

② larger protein (size > pore size) will be impended in their migration

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* General operation → separation
→ staining
→ detection and quantification
↳ blotting tech.

① Electrophoretic separation:

sampling &.

Any excess buffer is removed from support media by blotting

↓

Test sample is applied using comb. / plastic template

↓

Allowed to diffuse in media x 2 min

↓

gel is placed in the electrode chamber

↓

electrophoresis is performed at specific current, voltage and power

↓

gel is rinsed, fixed and dried

↓

stained and redried → scanned in densitometer

Note → If Isoenzymes are to be determined, substrate dye solution is incubated on gel to stain zone before fixing. and drying

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④ staining:

- use to visualise separated protein

fixing - protein are first fixed by precipitating them in gel ⊂ chemical agents eg, methanol / acetic acid
↓
prevent diffusion of protein out of gel when submersed in stain solution.

- comanon stains used in practice.

① Serum protein	Amidoblack CBB G-250 CBB R-250	640
② Iso-enzymes.	Nitrotetrazolium blue.	
③. DNA	Ethidium bromide. (fluorescent)	245 (Ex) 590 (Em)
Lipoprotein	Oil Red O sudan black B.	
CSF protein	Silver nitrate ↳ high sensitivity than Conventional dye	

③ Quantification :-

by densitometer.

↓

each ~~part~~ fraction of gel is moved past photometric optical system.

↓

Displays electrophoregram

↓

~~Integro~~ Integral area under each peak is calculated and reported as % of total or absolute conc.

④ Blotting techniques :-

to detect fragments of DNA, RNA and protein.

↙ ↓ ↘

Southern Northern Western

Electrophoretic separation of DNA or RNA fragment using by AGE

↓

Strips of nitrocellulose / by nylon membrane is laid over agarose gel

↓

DNA and its fragments are 'blotted' or transferred on to it by capillary, electro- or vacuum blotting.

↓

Hybridized & labeled, complementary NA probe.

↓

detected and identified.

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Use: (1) molecular biology for identifying particular DNA seq.
(2) determine the, position and no. of copies of a gene in genome.
(3) DNA typing.

* Technical and practical considerations.

(1) Sampling :-

Sample amount should be optimum.
↓
to achieve proper balance b/w sensitive measurement and resolution

ex → Albumin → 10% more conc. than. α1 α2 fraction as, globulin.
↓
Amount applied
↙ ↘
to prevent overloading of albumin — adequate to quantify

(2) Discontinuities in Sample applicat°
by ① dirty applicator
② uneven absorption by media.
③ Inclusion of bubble if sample is directly applied on media through pipette.

(3) Unequal migration rate :-
① Dirty electrodes → uneven applicat° of electric field.
② Uneven wetting of gel.
③ " " of wick if used.
④ Uneven thickness of gel.
⑤ gel stored for longer time (near heat
↳ unevenly dried

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(4) Distorted bands:-
- bent application
- air bubble incorporation during application.
- Over application.
- Inadequate blotting of sample
- Excessive drying of support
- Excessive wet gel

(5) Unusual / Artifacts:

(1) hemolysed sample → ↑ B-globulin
(2) Hemoglobin - haptoglobin complex
↳ Band b/w α_2 - B globulin
Also denatured protein ↑
(3) Fibrinogen band - at starting of electrophoregram
(4) Split albumin zone → Bis-albuminemia

(5) Grossly wide albumin zone
↓
dlt albumin bound medication
↳ acidic drug eg. warfarin, phenytoin

(6) Atypical bands:
dlt binding of Ig to (M) isoenzyme
↓
Abs interaction

* Types of electrophoresis:

① Slab gel electrophoresis
- Traditional method.
- Using a rectangular gel regardless thickness.
- Media = starch, gel agarose, polyacrylamide.
- gel cast on a sheet of plastic backing.
↓
Horizontal / vertical electrophoresis and submersion for cooling.

- Gel may be cast ~~c~~ with additives e.g.
① Ampholytes → create pH gradiant
↓
isoelectric electrophoresis

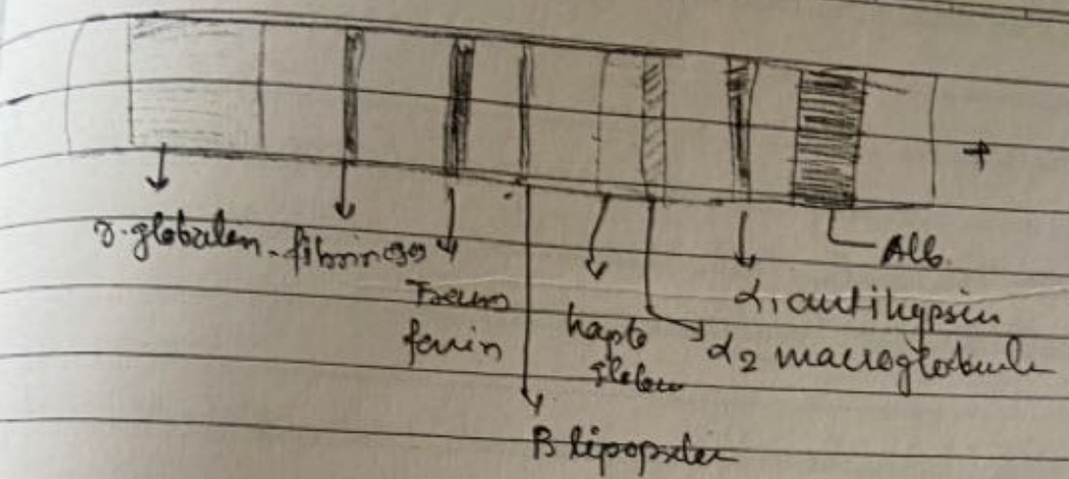
② SDS → denatures protein
↓
2-D electrophoresis

ordinary PAGE → only 5 protein bands

③ Disc Electrophoresis: - disc - 20 bands

Derived its name from Discontinuous electrophoretic matrix caused by polyacrylamide / starch gel. that differs in composition and pore size
↓
3 gel system prepared
① thin, small pore, separation gel
② thin, large pore, spacer gel
③ thin, large pore monomer → sample is applied
↓
protein ions stack up on separation gel.
↓
improves resolution + concentrate protein
↓

simplified drawing of PRE. pattern.



Note:

3 major class of protein = Ele

① Albumin
② globulin → α1 globulen → α1 antitrypsin
→ α2 globulen → α2 macroglobulinen
→ haptoglobin
→ β globulen → β microglobulin
→ transferrin
→ β lip-protein
→ γ globulen
③ fibrinogen

② Isoelectric Focusing electrophoresis (IEF)

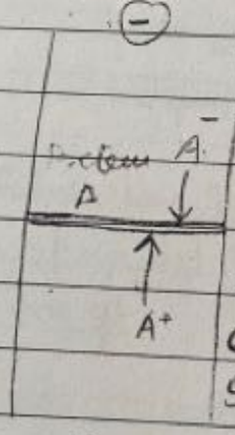
* Principle:-

Separation of amphoteric compounds, like protein, in ped gel solution in medium possess stable pH gradient.

→ Protein become focused at point where pH of gel matches its PI.

↓

very sharp protein zone, as the region associated w pH is very narrow.



Example:-

pH=8 is the pI of protein A

↓

protein A will be focused at pI = pH=8 zone

↓

will attain net zero charge

A^0

when migrate towards cathod, it will attain -ve charge (A^-)

↓

A^- will migrate back. bez of electrophoretic forces.

when diffuse towards anode, it will attain +ve charge

↓

A^+ will migrate back to pI zone bez of electrophoretic force.

pH gradient :-

created ĩ carrier ampholyte

↓

group of polyaminocarboxylic acid

↓

having slightly different pH values and mw 300 - 1000.

↓

carrier ampholyte added to medium

↓

Individual ampholyte reach to their pĩ during electrophoresis.

↓

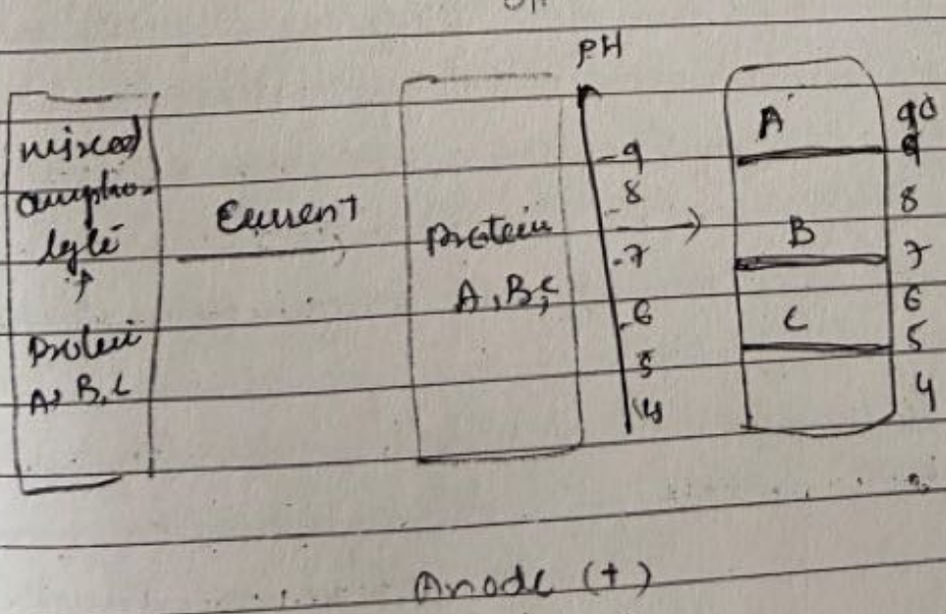
establish narrow buffered zone ĩ stable but slightly different pH

↓

Natural pH gradient

↓

through which protein molecules migrate and stop at their pĩ.



shown in figure,
Anode is surrounded by diluted acid
Cathod " " alkaline
↓
current passed, focusing occur
↓
Most rvely charged amphotyli carrier and protein will be found near anodal end, and vise versa.

- high conc. of amphotyli used, so high voltage power source needed (2000v).

* Modificat^{n} of IEF

→ IPG : IEF - Immobilized pH gradient IEF (4)
↓
Immobilized pH gradient is produced in gel before sample applicat^{n}.
↓
Improve resolut^{n} and reproducibility

→ PAGE - IEF → widely used. as free of endosmosis
Although large pore size needed, migrat^{n} is not affected by molecular sieving effect.
↓
AGE - IEF → advantage of large pore sized.

→ Off. gel technique. → Separat^{n} in free solut^{n}.
↓
Sample containing amphotyli is loaded in linear series of well
↓

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each well is separated by semipermeable membrane and pH gradient on other contact i pH gradient strip

↓

protein is separated in to different well depending on their pI.

↓

further separated by 2D Electrophoresis

↓

used in human proteome.

(4) Isotachophoresis (ITP)

Iso = same tacho = speed phoresis = separation

- Complete separation of small ionic substance i same rate. i no gap/overlap
- Same occurs in stacking gel of PAGE
- Sample is placed b/w
  - (1) leading electrolyte sol. (Cl⁻) → that moves faster than sample
  - (2) trailing sol (glycine) → that moves slower than sample

↓

once, after moving component separates completely from slower moving one

↓

Any further separation will create a region of depleted charge and T

↓

lead to ↑ local voltage in that region.

↓

causes slower moving component to migrate faster and fill the gap.

↓

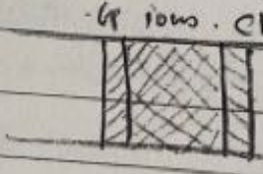
So, all ions migrate at same rate. ~~in~~ zones ~~that~~ differ in size depend on their ~~original~~ original conc.

↓

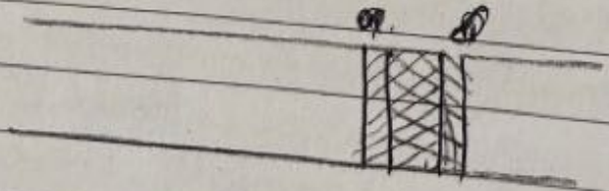
Zone size is determined by measuring UV absorbance, temp difference or conductivity as sample passes detector.

Use → in separation of small ions, anion, cation, AA, nucleoside-tide, protein.

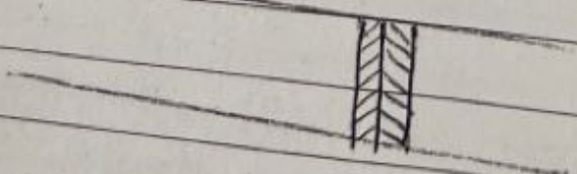
Sample in capillary. ∴ leading and tailing ions (Cl⁻) (other than glycine)



Cl⁻ moves faster creating gap.



gap will be detected as zone of ↑ local voltage created



Separated ionic substance ∴ No gap / overlap ⇒ detected

Pulse field electrophoresis

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Power is applied to diff. pair of electrodes or electrode array.

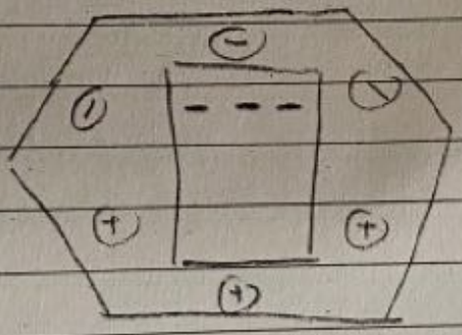
↓

electrophoretic cycle field is select. b/w 2 direct^n.

use → for sep^n separat^n of very long molecule e.g. DNA frag. > 50 kb. (typing chart of Bacterial DNA)

↓

can not be resolved by small. pores in agarose / polyacry.



→ Net migration will be in forward direct^n. in case of DNA sep.

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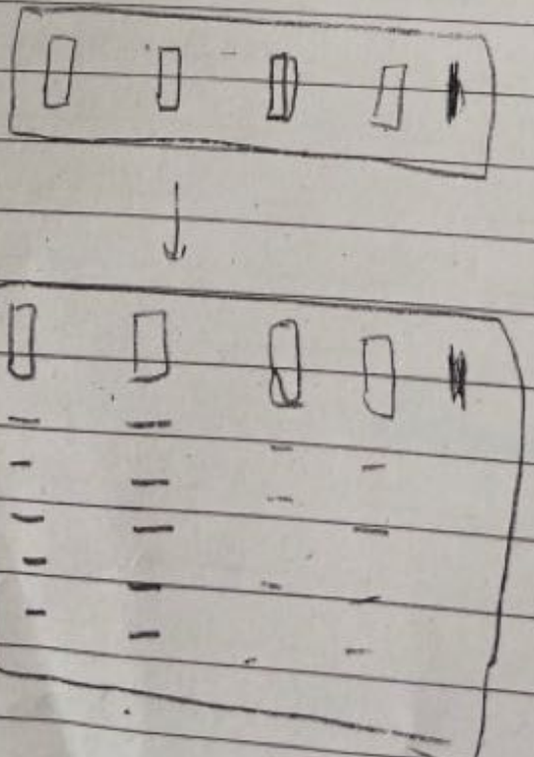
⑥ 2-D Electrophoresis :-

Separation is done by ~~first~~,
charge dependent IEF in first dim.
↓
F I B.
Size gel.
~~Weight~~ - dependent electrophoresis in 2nd dimension eg. PAGE.

Procedure :

1st dimension done is 130 x 2.5 mm tube & covers pH range of 3-10 unit
↓
After completion, gel is extruded from tube and placed in contact of polyacrylamide gel slab co. that incorporates SDS
↓
Denaturing protein by x disulfide bon



use:

(1) in field of proteomics.
   ↳ study of protein in dif. genes
   ↳ study of protein content of cell.

(2) study of human genome mutation and DNA of various bacteria and tumor cell.

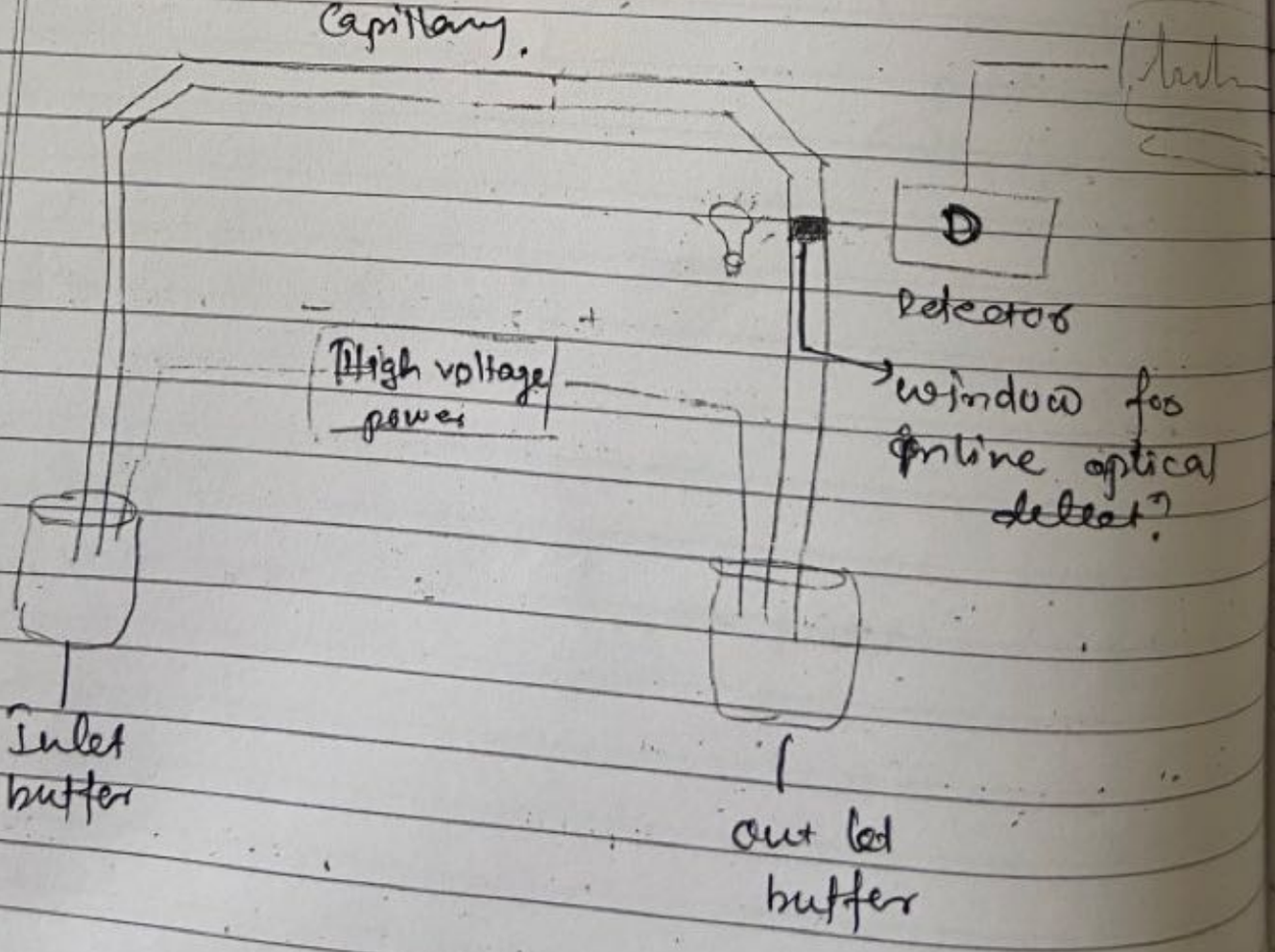
⑦ Capillary Electrophoresis (CE)

classic techniques of ZE, ITP, IEF and gel electrophoresis carried out in a small-bore (10-100 μm internal diameter) fused silica capillary tube.

→ 2 advantage
① Ease of automation → No gel, No staining, inline detect" → high sc
② Ability to apply high voltage than conven electrophoresis. → easy cooling
↳ fast separat" → ↓ TA)

- use:- for separation of inorganic ions, AA, organic acids, drugs, vitamins, porphyrins, carbohydrate, protein, DNA

* Instrumentation



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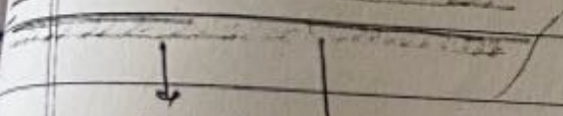
The capillary format:-

- made from fused silica coated with thin exterior covering of polyimide.
  - → provide strength & flexibility.
  - → Removed from small portion, close to terminal end for online optical detect^{n}.



↓ polyimide covering
→ Window for inline detect^{n}.

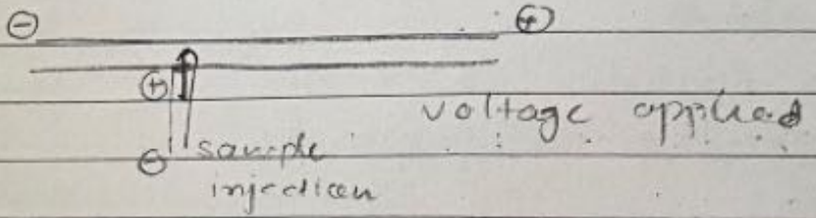
- Inner diameter – 20 - 150 lem
  outer " 150 - 375 lem.
  length - 20 cm to several meter.

- Capillary shape
  - Cylindrical ↓ ○ → optical detect^{n}.
  - Non cylindrical ↓ □ ▭ → rectangular capillary provide flat surface → more responsive to optical detect^{n}

(2) Sample Injection:- 1-50 nL loaded by 2 methods

(i) Hydrodynamic injection:
↓ applying
Sample is injected by positive pressure at inlet vial and vacuum at out vial.

② Electrokinetic injection: (EK)



Sample is injected by applying voltage for timed interval.

↓

magnitude of ~~the~~ voltage → depends on analyte and buffer
→ 1/3 - 1/5th times the field strength for separation.

→ EK injection favours preferential movement of more electrokinetically mobile analyte into capill

③ Direct Detection:

- ~~Online~~ optical detection through detection window created by burning outer polyimide layer and cleaning capp capillary & methanol

→ ~~Inner~~ diameter

→ Optical detection done by
(1) Photometric absorbance → UV visible photometr
(2) Refractive index.
(3) Fluorescence. 4mc.

→ Inner diameter of capillary = optical

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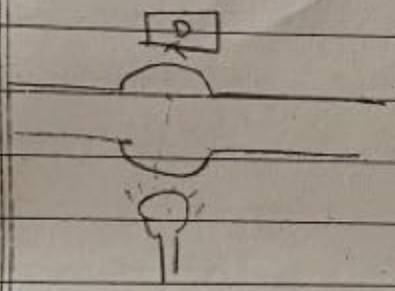
↓

20 - 100 lel diameter limits ↑ UV. visible absorbance detection limits ↓ conc. 10^{-8} to 10^{-6} mol/L.

* Improving limit of detection :-
by (1) ↑ diameter of OPL
(2) using more sensitive optical teq.
(3). preconcentrating sample.

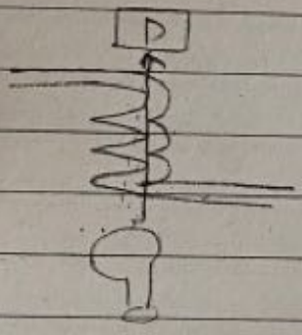
(1) ↑ OPL :-

Bubble cell



glass blown expansion of internal diameter of capillary.

2 - geometry



(2) Sensitive optical detectors :-
includes (1) fluorescence.
(2) chemiluminescence
(3) Refractive index.
(4) mass spectrometry
(5) Electrochemical detect^{n}. Used for homocystein & epinephrine

② Online Sample Preconcentration:

⊛ Simplest method is to introduce stacking effect

↓

dlt ionic strength difference b/w sample matrix and separating buffer

↓

Red electrophoretic mobility of sample ion in higher conductivity environment

↓

stacking of sample component into a buffer zone

↓

Enhancement in sample concentration

Isotachophoresis →

③ Focusing technique

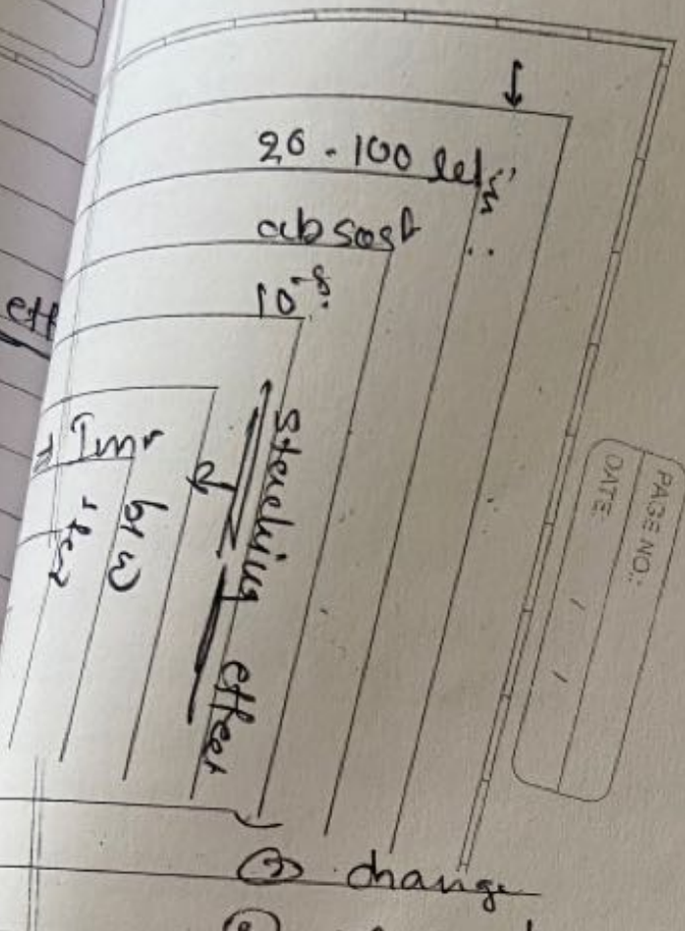
↓

Based on pH difference b/w sample plug and separation buffer.

↓

Similar to isoelectric focusing.

→ useful in analysis of peptide bez of their relative stability over wide range of pH.



face effect
reflects

(cab), moderate-
power)

↓ with ↑ heat
o to

temp gradient
viscosity

③ change
④ zone broadening

→ Capillary electrophoresis, electric field applied is extremely high (30000V)

↓

Heat produced is prevented by effective dissipation of heat by ① forced air convection or liquid cooling ②

↓

Both can be possible bec of to narrow bore of capillary.

~~① other attempts~~

→ Heat product" is dependant on

① Capillary diameter → ↓ diameter - ↓ heat generat"

↓

less desirable as ↓ing diameter will ↓ detect" limit of UV mea-

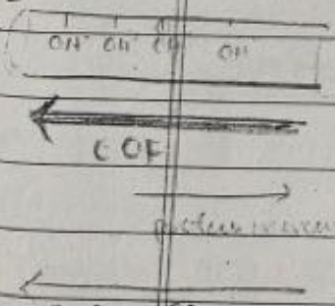
② Voltage applied → ↓ strength of field will
↓ heat generated
↓
↓ resolution → less desirable

③ Buffer type
④ Concentration
} → attempts should be made to alter these parameters before applying applied field.

⑤ Surface effect → Electroendosmosis
Adsorption

capillary wall → made up of fused silica
↓
has SiOH group

(1) Electroendosmosis :-
↳ primary driving force for separation in capillary electrophoresis (CE)
↓
consequence of surface charge on solid support (negative charge)
↓
ζ is determined by ionization of silanol (SiOH) group that populate it.
↓
Interaction of +vely charge buffer species & surface anion generate a layer of mobile cation
↓
ζ moves them towards cathode when voltage is applied → solvent



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↓

This will induce very strong EOF that mobilize all analyte in same direction regardless of their charge

↓

Separation is achieved bcoz of diff. in electrophoretic migration rate of analyte

- EOF depends on
  1. Type of electrolytes used
  2. pH
  3. ionic strength
  4. use of additives (surfactant / organic solvent)
  5. polarity and magnitude of applied electric field

→ Flat flow profile

(2) Adsorption through surface:

large surface area-to-volume ratio

↓

Adsorption of analyte on to surface of inner wall

↓

Due to typically polar/ionic solutes and -vely charge inner wall through

↙ ↘

Ionic interact" (SiOH) | hydrophobic interact". (Siloxane)

↓

causes 'peak tailing'

⇒ These inner wall effects can be minimized by chemical ~~Ht~~ of inner wall

↓

base (0.1 - 1.0 mol/L NaOH). wash removes adsorbates and rejuvenate surface

↓

~~Hb~~ flushing & running buffer ~~& prevent drastic fluctuat" in pH~~

→ following components are fabricated on surface of microchip, using photolithographic process.

① Separation channel
② Sample injection channel
③ reservoir
④ Sample preparation
⑤ pre column / post column reactor

⇓

Multifunctional 'Integrated' analytical device embedded in single monolithic substrate is possible.

⇓

classic cross T design of single channel microchip contains involves.

cyclohexanoic acid.

→ Buffer solution - required in very lower volume compared to CE

• Sample injection - nL to pL in volume
- CE sample injection mode is used.
- Several hundred of voltage applied to Sample injection and sample waste reservoir

↓

Migrate sample to cross-T site

• Separation voltage - 1-4 kV applied to separation channel

↓

Separation

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ace of

. detection - throug UF (laser induced fluorescence)
↓
easily implemented in planer configuration of microchip.

- Limit of detect^{n} = 10^{-18} to 10^{-15} moles.

* Fabrication of microchip:
- microchips are constructed from
① glass
② silicon } etc.
③ Variety of polymers e.g. plastic
④ Silicon like materia e.g. polydimethylsiloxane

thing glass → Solution of hydrofluoric acid used to etch desired structure in glass.
↓
produces smooth wall, U-shaped channel bec of decreased and lateral etching by etch solution

* Molecular diagnostic using microchip.

Ds DNA is bound to fluorescent intercalaters
↓
separation by microchip electrophoresis
↓
easy detect^{n} by UF & high sensitivity

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* ~~Modes of operation~~ :-

* Microchip electrophoresis

Principle of molecule separation is same as capillary electrophoresis.

Advantage over CE

1. High ~~spte~~speed - 10 fold faster than CE
2. Simplicity
3. Capability for chip integration of multiple function
4. Automatation.

* Instrumentation