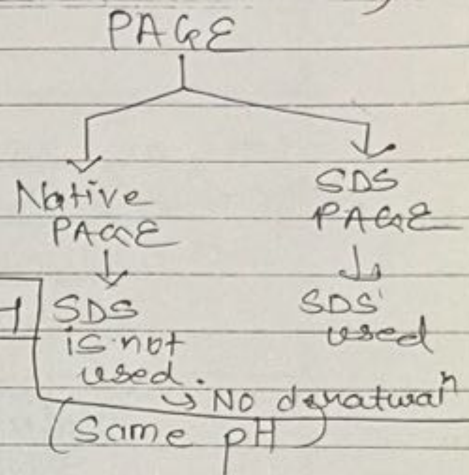
(Laemmli Buffer system) SDS PAGE :— → useful to assess purity of protein
(Discontinuous Buffer system is used)



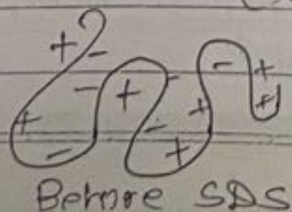
* **Steps :—**

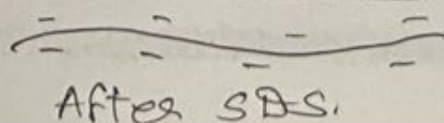
① Sample Buffer formation :—

→ requires Tris 0.5 M, 6.8 pH
↓
As stacking gel pH is 6.8 (Same pH)

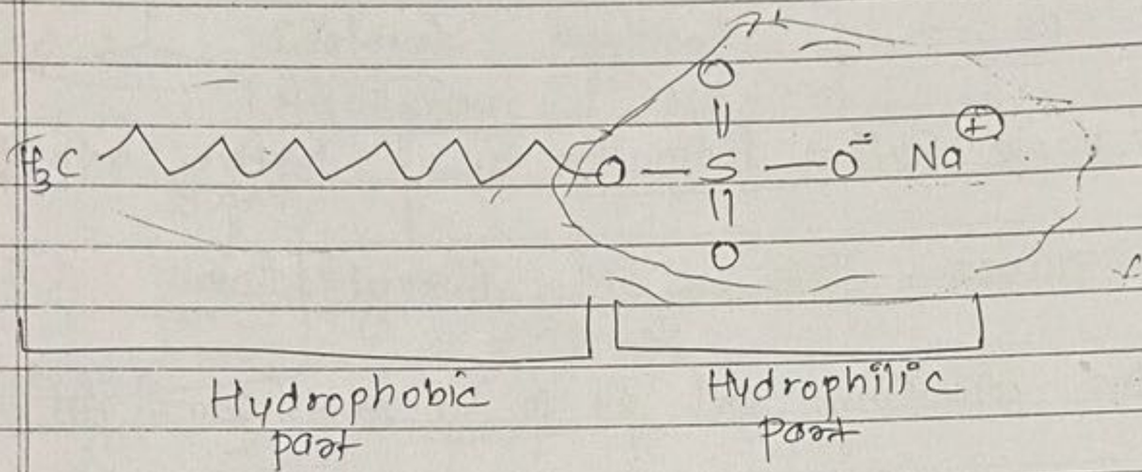
→ SDS → to make protein denature
→ Glycerol → to make sample heavy, so will not diffuse
→ EDTA → used to chelate metal ions
↓
which is required for enzyme activity (Protease)
↓
So enzyme will not work & degradation of protein will not occur
→ BPB → Tracking dye.

② SDS :— anionic detergent → dissolves hydrophobic molecule (have -ve charge)
↓
Binds c̄ polypeptide
↓
make it linear & impart a -ve charge
→ denaturation of protein (only 1° structure remain)


Before SDS


After SDS.

SDS structure :-



(2) Formation of 1% Agarose gel
↓
To prevent leakage (For sealing purpose)
↓
also prevent collapsing of separating gel.

(3) Separating gel :-

contains Acrylamide - Bisacrylamide
↓
Copolymerization of acrylamide & Bis-acrylamide
↓
Polymerization is initiated by a free radical generating system
↓
Polymerization is initiated By APS & TEMED
↓
TEMED :- accelerates the rate of formation of free radicals from persulfate
↓

↓

Persulfate free radicals ~~c react with~~ converts acrylamide monomer to free radicals ~~from persulfates~~ c̄ reacts with unactivated monomer to begin the polymerization chain reaction.

↓

The elongating polymer chains are randomly crosslinked by bis

↓

resulting in a gel c̄ a characteristic porosity which depends on the polymerization conditions. & monomer concentrations.

⟹ Riboflavin (Riboflavin-5'-phosphate)

↓

also used as source of free radical.

↓

can be used in combination c̄ TEMED & ammonium persulfate

↓

Riboflavin —(+nce of light & Oxygen)→ Leuco form

↓

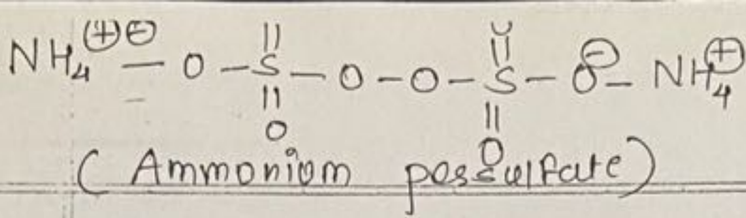
Active Form

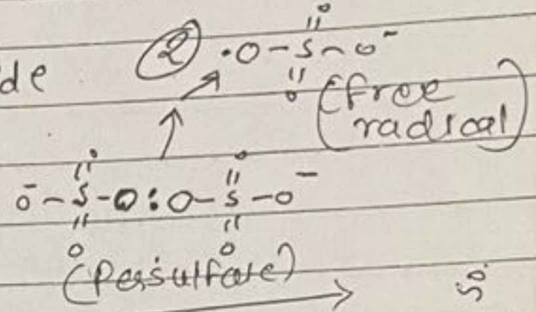
↓

Initiate polymerizati

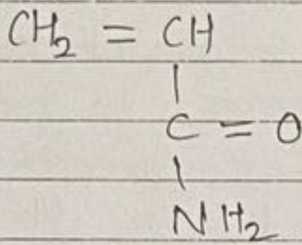
↓

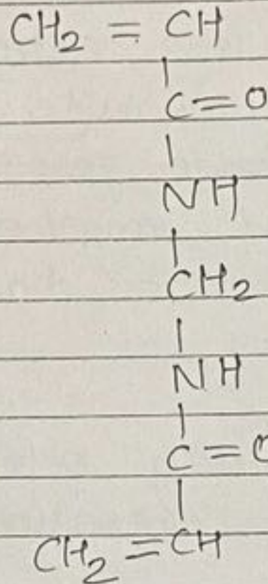
k/a "Photochemical polymerization"

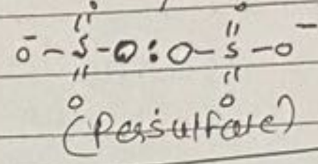


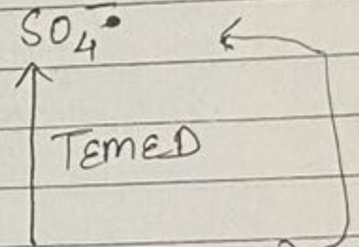


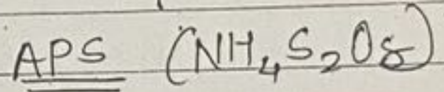
Acrylamide   Bisacrylamide

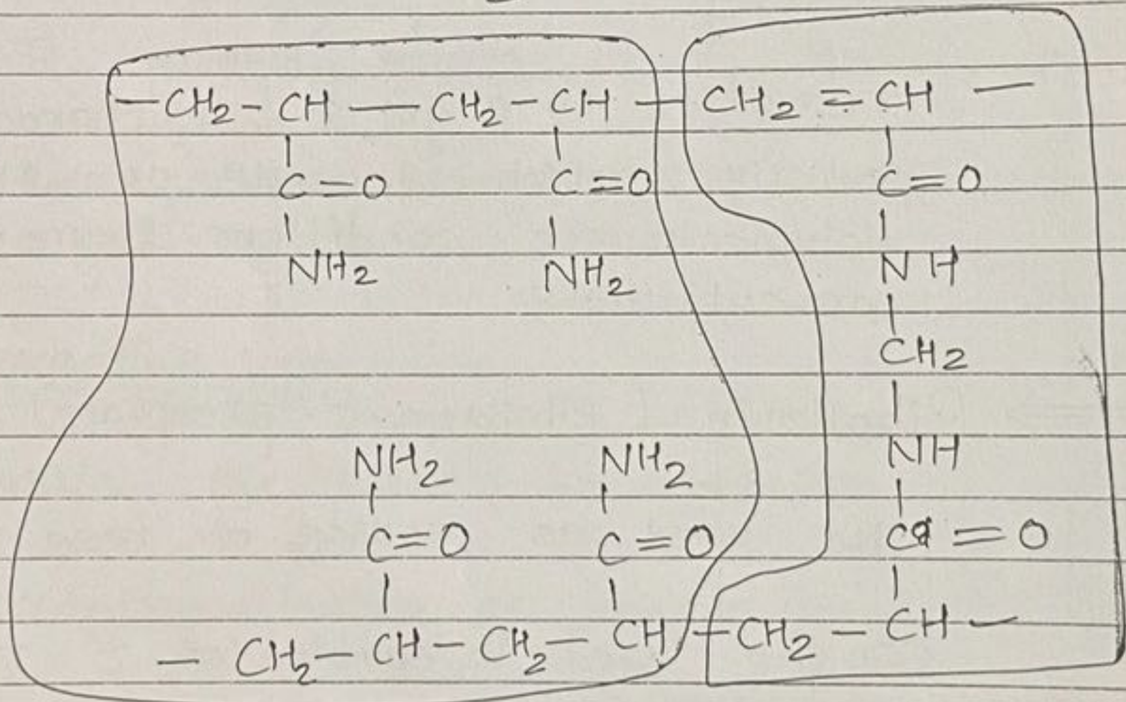








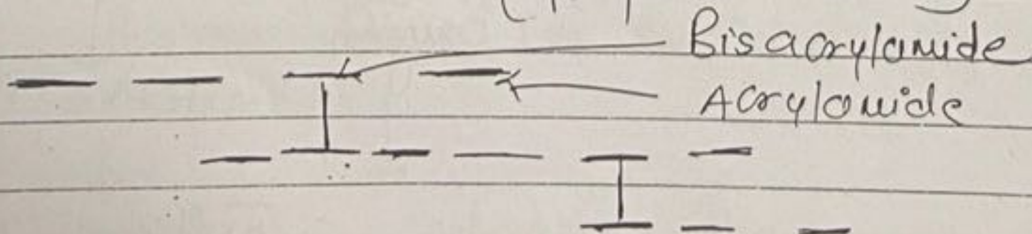




Bisacrylamide

Acrylamide

(Polymerization)



* Majority of Heat is released (generated) at the last stage
↓
So By using temperature prob → we can say that the polymerization occur

* Interfering compounds :-

(A) Acrylamide → purity :-

(a) Acrylic acid :-

↓

deamidoⁿ product of acrylamid.

↓

Can copolymerise c̄ acrylamide & Bis.acrylamide

↓

Thereby conferring ion exchange properties on the resulting gel

↓

lead to change in local pH in the gel

↓

Causes artifacts such as aberrant relative mobility, precipitation of some proteins & nucleic acid, smearing of bands

→ So in acrylamide - acrylic acid should. below 0.001% (w/w)

↓

determined by direct titraⁿ
→ supported By both conductivity & pH measurement.

(b) Linear Polyacrylamide :-

↓

Autopolymerizoⁿ during the production, processing / storage of marginally pure acrylamide

* Silica → water absorbant
  → So put silica in Amm. persulfate
  it takes water from APS

↓

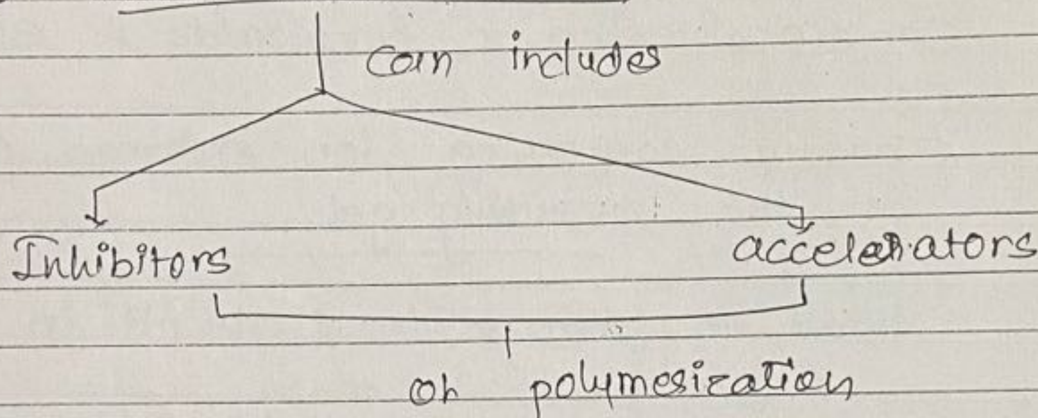
result in trace of linear polyacrylamide

↓

Affect polymerization.

So

(c) Ionic Contaminants:-



→ Metal ions such as copper
↓
Inhibit gel polymerization.

→ Metal → also poison enzymes.
↓
Alter the relative mobility of metal binding protein such as calmodulin
↓
Inhibit digestion of electrophoretically purified nucleic acid by restriction & modification enzymes

O_2 → Binds c̄ polymer → so Radical on polymer
Binds c̄ O_2 → O_2 Become Radical → inhibit
→ act c̄ TEMED & APS
→ O_2 takes e⁻ from APS radical & acrylamide & Forms radical But this radical is not powerful

* Role of SDS:

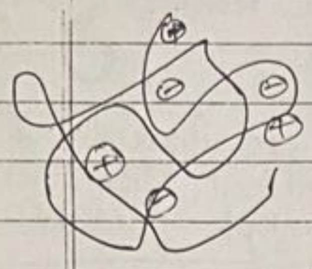
DTT : ~~Dithiothreitol~~ Dithiothreitol } Reducing agent
B-ME : Beta mercaptoethanol }
(2-mercapto ethanol)
↓
Breaks the disulfide Bond (Interchain/ Intrachain):

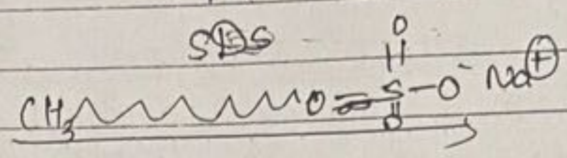
→ SDS c̄ reducing agent
↓
disrupt tertiary structure of proteins.
↓
forms linear protein molecule.

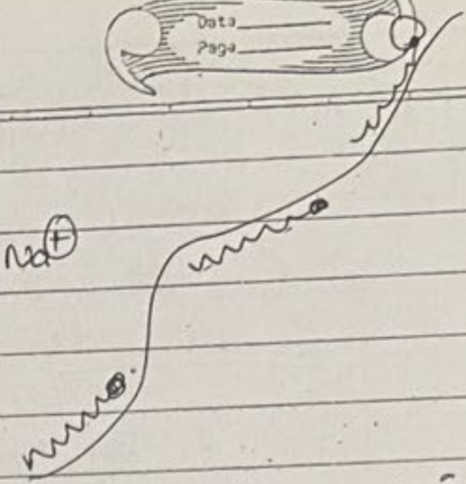
→ SDS (-vely charged detergent)
↓
coats protein c̄ uniform -ve charge
↓
c̄ Masks the intrinsic charge on R-group.

→ Approx. 1.4 gm/1 gm of protein is require [1 molecule of SDS/2 peptide Bonds)

→ Molecular wt ∝ length of linear protein molecule
∝
-very charge on protein ∝ No. of SDS attached to the linear molecule of protein







Foled protein $\bar{c}$ intrinsic charge.

Denatured protein $\bar{c}$ uniform -ve charge.

* Gel Formation :-

→ Polyacrylamide gel matrix used
↓
~~forms~~ prepared by monomer of acrylamide & Bisacrylamide
↓

Adv :- ① Chemically Inert :- will not interact $\bar{c}$ protein in the sample,
② easily reproducible $\bar{c}$ diff. pore size

→ Why not only acrylamide useful ?
↓
if used alone — would polymerize into long strand
↓
NOT A POROUS GEL

O_2 Removal → degassing → vacumm
→ ultrasound
→ N_2 gas → N_2 has low

O_2 → O_2 follows conc. gradient

↓ can be used Riboflavin → so Riboflavin Binds c̄ O_2

→ Bis Acrylamide cross links the acrylamide gel.

* Neutral / Basic pH is required for optimum action of TEMED

↓

Free Basic Form of TEMED is required for initiation of polymerization.

↓

So in neutral / Alkalin pH → TEMED is in the Free Basic form

↓

Can take H^+ & Protonated Form is formed.

→ In acidic pH

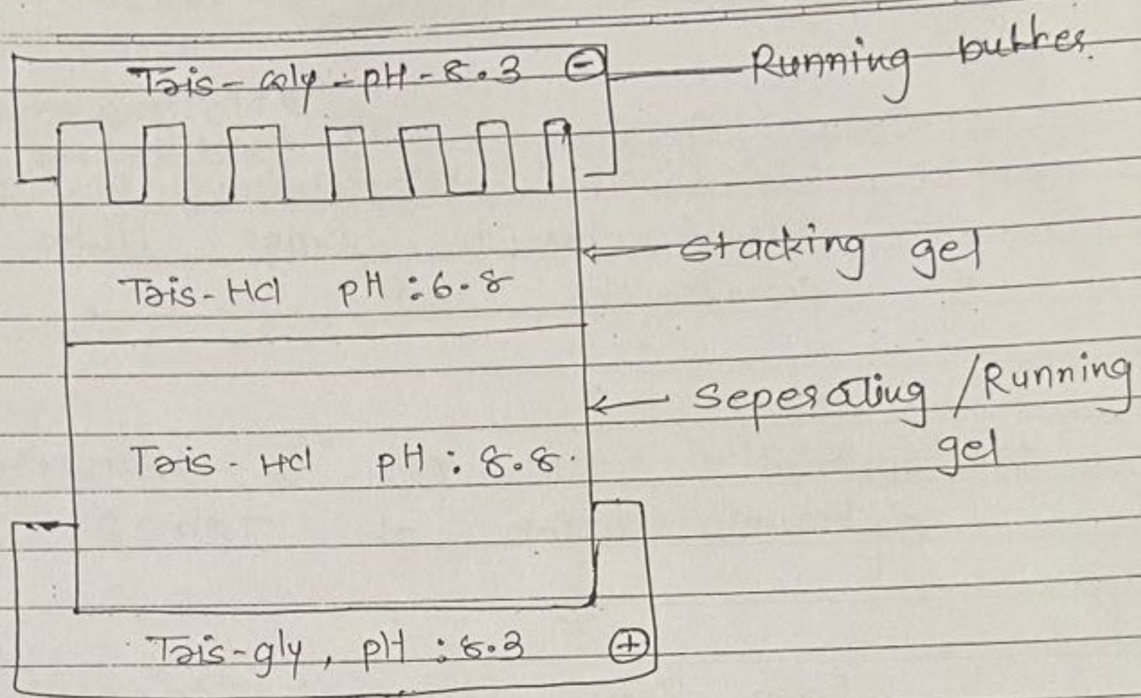
↓

TEMED already forms protonated

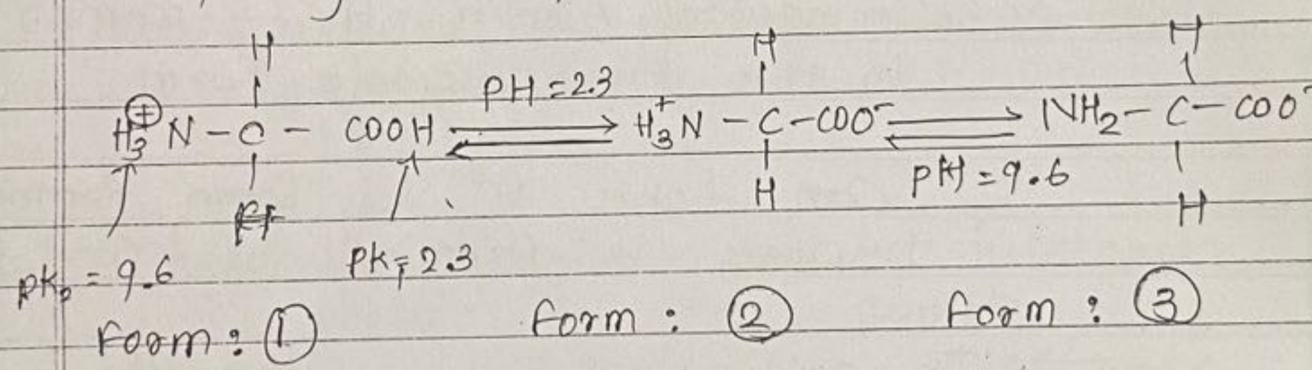
↓

So can't take e^- from APS & make APS - radical.

*



→ Glycine exist in 3 diff charge state depanding on pH.



→ When the power is turned on

↓

-vely charge glycine ion in the pH : 8.3 buffer are forced to enter the stacking gel

↓

stacking gel, pH : 6.8, glycin become nutrally charged

↓

Glycine moves very slowly in electric field

↓

Cl^- ion from Tris-HCl in stacking gel, move much more quickly in the electric field

↓

They form an ion front that migrate ahead of glycine

↓

These will create a narrow zone c̄ a steep voltage gradient, that pulls the glycine along behind it

↓

The proteins in the gel sample have mobility intermediate B/w that of glycine & Cl^-

↓

So when the 2 fronts sweep through the sample well, the proteins concentrated into the narrow zone b/w Cl^- & glycine front

↓

These process occus untill it hits ~~running~~ separating gel.

↓

↓

In Running gel (pH : 8.8) (separating gel)

↓

Glycine molecules are mostly -vely changed

↓

Can migrate much faster than proteins

↓

So the protein are dumped in a very narrow band at the interface of the stacking & running gel.

↓

Running gel has ↑ acrylamide concentraⁿ which slows the movement of protein acc. to their size

↓

Seperation Begins.

* Why stacking gel requires?

↓

If only running gel is used the proteins will enter in gel at different times

↓

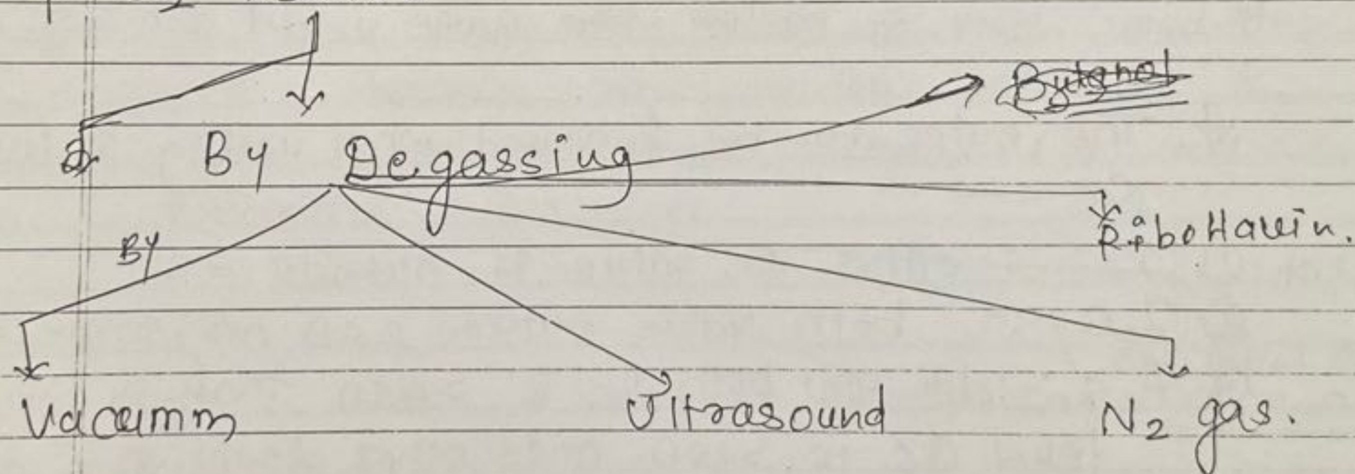
resulting in very smeared Bands.

↓

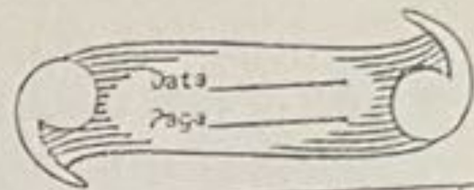
stacking gel ensures that all of proteins arrive at running gel at same time

⟹ ~~Why~~ SDS is +nt in all gel & Buffers
↓
To make the ~~sampl~~ protein in the sample linear -vely charged & denatured.

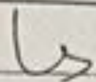
* O_2 Removal



⟶ also Riboflavin can be used c̄ APS & TEMED
↓
Riboflavin Bind c̄ O_2 so O_2 will not bind c̄ APS & TEMED



SDS - PAGE :-



To estimate moleculas wt or protein By putting standard.

NABL 112 (clause 5.6) Page-2