Overview of techniques in protein purification:

- i) Structural biology – Tertiary/Quaternary structure of protein molecule
- ii) Functional assay

→ Structure-function correlation ⇒ Functionally active structure of protein

➢ Limitation of molecular spectroscopic techniques in the analysis of protein structure:

Spectroscopic signals are chromophoric group specific not molecule specific

Absorption spectroscopy: peptide bond ~ 220nm ; aromatic amino acid residues ~ 280 nnn

Fluorescence spectroscopy: Tyrosine and Tryptophan (intrinsic fluorescence)

External flurophore

Nuclear Magnetic Resonance: ^{1}H; ^{13}C; ^{15}N signal

➢ **Protein must be purified**

Cell lysis (ultra sonication/lysozyme) → lysate → centrifugation to separate membranes

↓

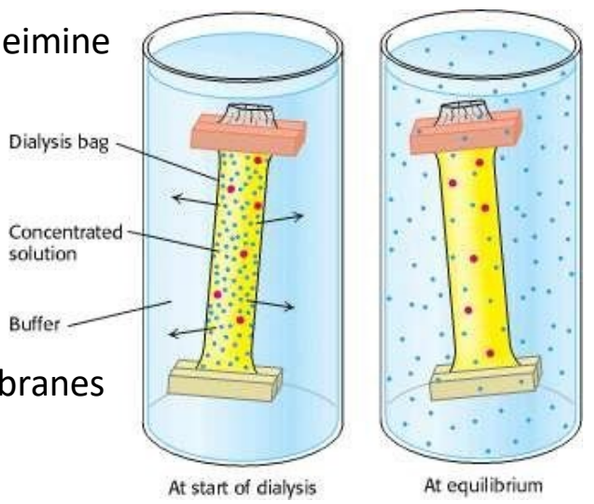
Precipitation of nucleic acids – Strptomycin/Protamine/Polyethyleneimine

↓

Centrifugation to separate nucleic acid pellet

↓

Dialysis (small molecules can pass through the pores of cellulose membranes



Gel Electrophoresis:

Electrophoresis – Molecules with net charge move in an electric field

⇒ Offers a powerful means of separation

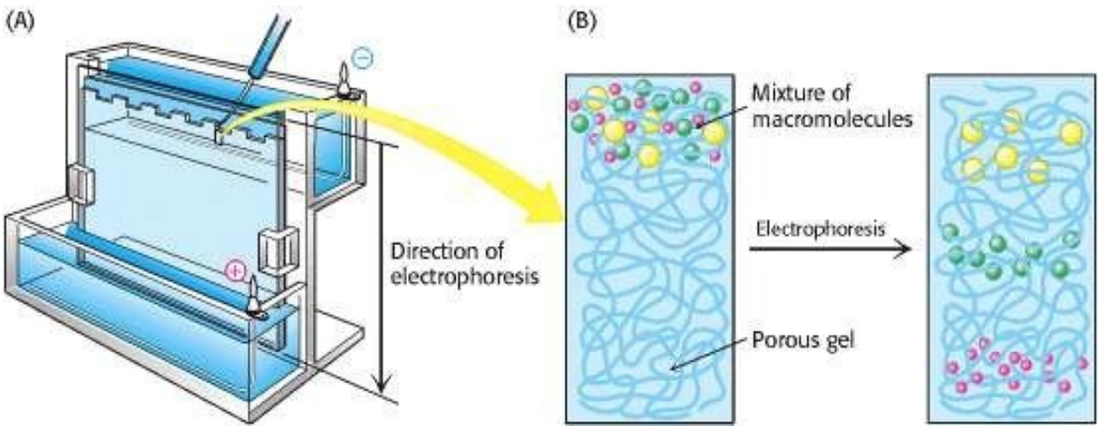
Velocity of migration of protein molecules in an electric field depends on:

Electric field strength (E), Net charge on the protein molecule (z) and Frictional coefficient (f)

Electric force, Ez, driving the charged molecule toward the oppositely charged electrode is opposed by the viscous drag, fv, arising from friction between moving molecules and medium

$$v = \frac{Ez}{f}$$

Electrophoretic separation are carried out in gels which serve as a molecular sieve that enhances separation. Molecules that are small compared with the pores in the gel readily move through the gel, whereas molecules much larger than the pores are almost immobile. Intermediate size molecules move through the gel with various degree of velocity. Electrophoresis is performed in a thin, vertical slab of polyacrylamide. The direction of flow is from top to bottom.



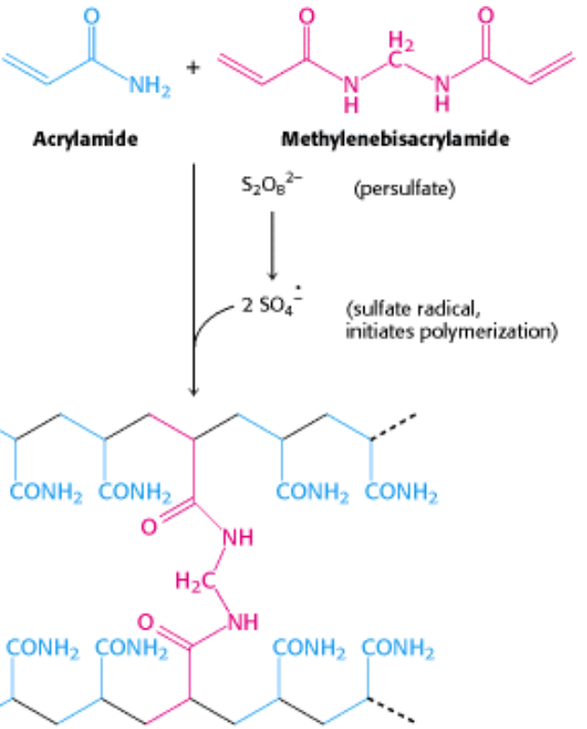
Polyacrylamide gels, formed by the polymerisation of acrylamide and cross-linked by methylenebisacrylamide, are choice supporting media for electrophoresis as it is chemically inert and readily formed.

Proteins are separated on the basis of mass by electrophoresis in a polyacrylamide gel under denaturing condition:

- Mixture of proteins is dissolved in a solution of sodium dodecyl sulfate (SDS), an anionic detergent, to disrupt all noncovalent interactions in the native protein
- Mercaptoethanol (2-thioethanol) is added to reduce disulfide bonds
- Anions of SDS bind to main chains at a ratio of about one SDS anion for every two amino acid residues
- The complex of SDS with denatured protein has a large net negative charge that is roughly proportional to the mass of the protein
- The negative charge acquired on binding SDS is usually much greater than the charge of the native protein

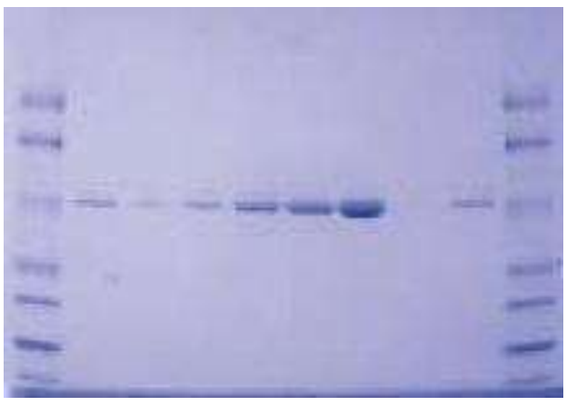
➢ Polymerisation is initiated with freshly dissolved ammonium persulfate together with a free radical scavenger, TEMED

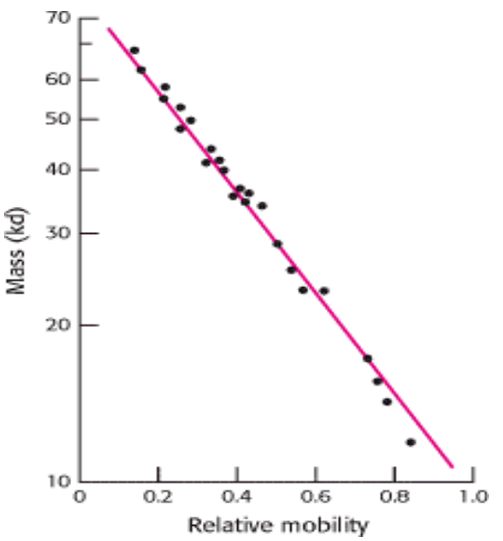
➢ The mobility of most of the polypeptide chains under these conditions is linearly proportional to the logarithm of their mass



Proteins in the gel can be visualized with silver or coomassie blue, which reveals a series of bands

SDSPAGE





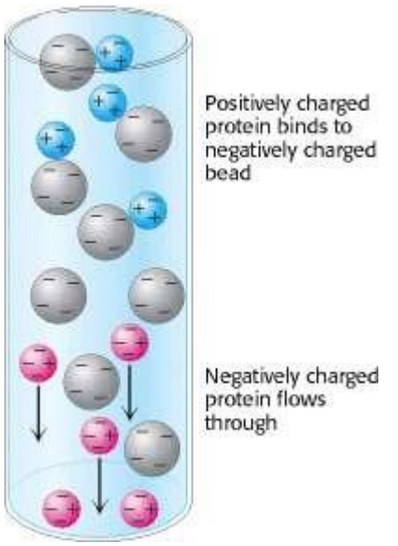
Ion-exchange chromatography:

Proteins can be separated on the basis of their net charge

➢ A positively charged protein binds to a column of beads (stationary phase) containing carboxylate groups whereas a negatively charged protein will not bind

✓ Positively charged proteins (cationic proteins) are separated on a negatively charged carboxymethyl-cellulose (CM-cellulose) columns

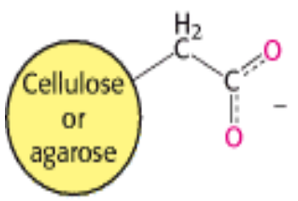
✓ Negatively charged proteins (anionic proteins) are separated on positively charged diethylaminoethyl-cellulose (DEAE-cellulose column)



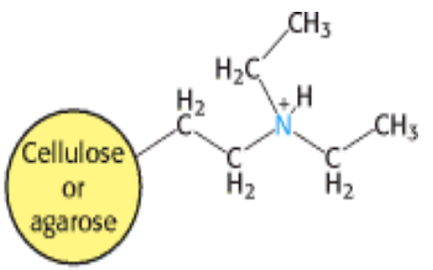
Positively charged bound protein can be eluted out of the column by increasing

the concentration of soduim chloride or another salt in the eluting buffer as sodium ions compete with the positively charged groups on the protein for binding to the column

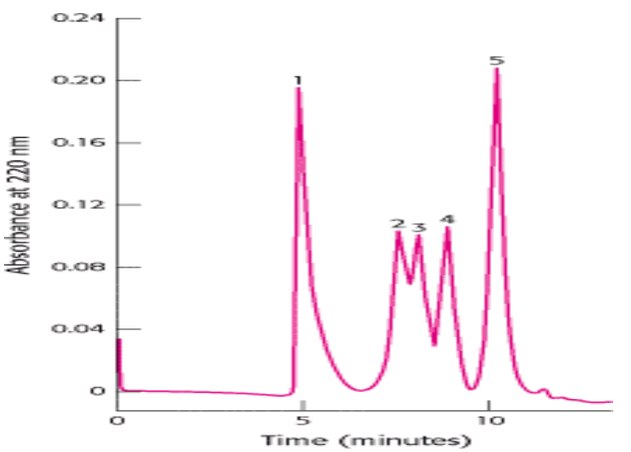
Proteins with low density of net positive charge will elute first, followed by those having high charge density



Carboxymethyl (CM) group
(ionized form)



Diethylaminoethyl (DEAE) group
(protonated form)



Gel-filtration chromatography:

➢ Proteins are separated on the basis of the size

Sample is applied from top of a column consisting porous beads made of an insoluble but highly hydrated polymer such as dextran or agarose.

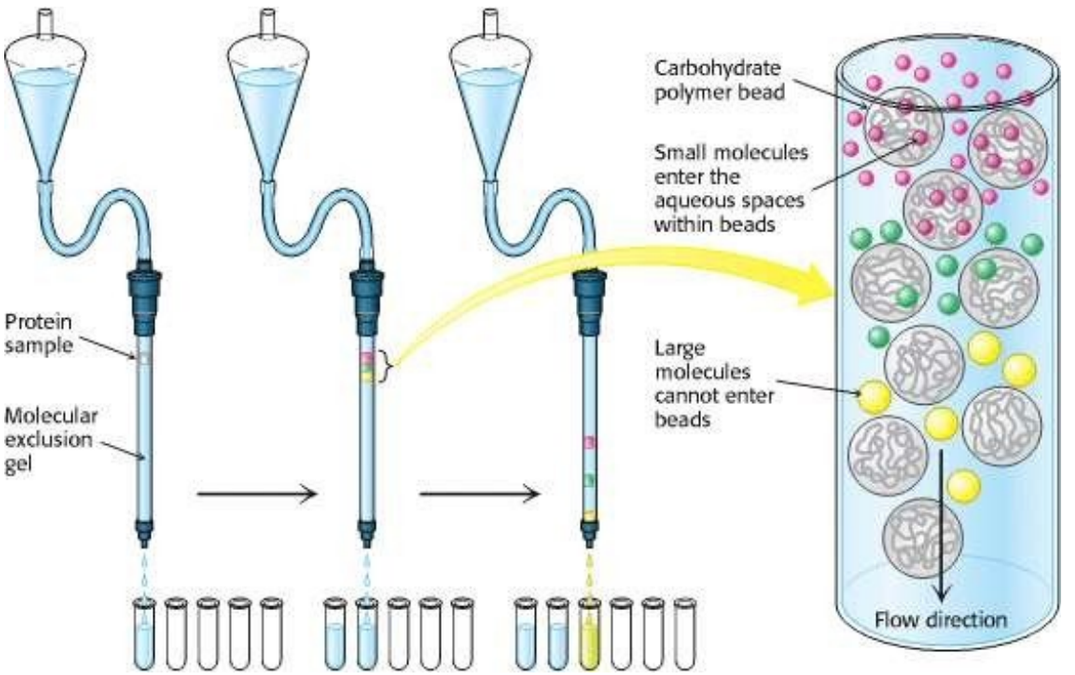
Sephadex, Sepharose, Bio-gel are commonly used commercial preparation of these beads, typically 0.1 mm in diameter

Small molecules can enter these beads, but large ones cannot ⇒ small molecules are distributed in the aqueous solution both inside the beads and between them, whereas larger molecules are located only in the solution between the beads

✓Larger molecules flow more rapidly through this column and emerge first because smaller volume is accessible to them

✓Molecules that are of a size to occasionally enter a bead will flow from the column at an intermediate position

✓Small molecules will exit last



Affinity chromatography:

➢ High affinity of proteins for specific chemical groups

Example: Concanavalin A – has high affinity for glucose

Concanavalin A can be purified by passing a crude extract through a column of beads containing covalently attached glucose residues

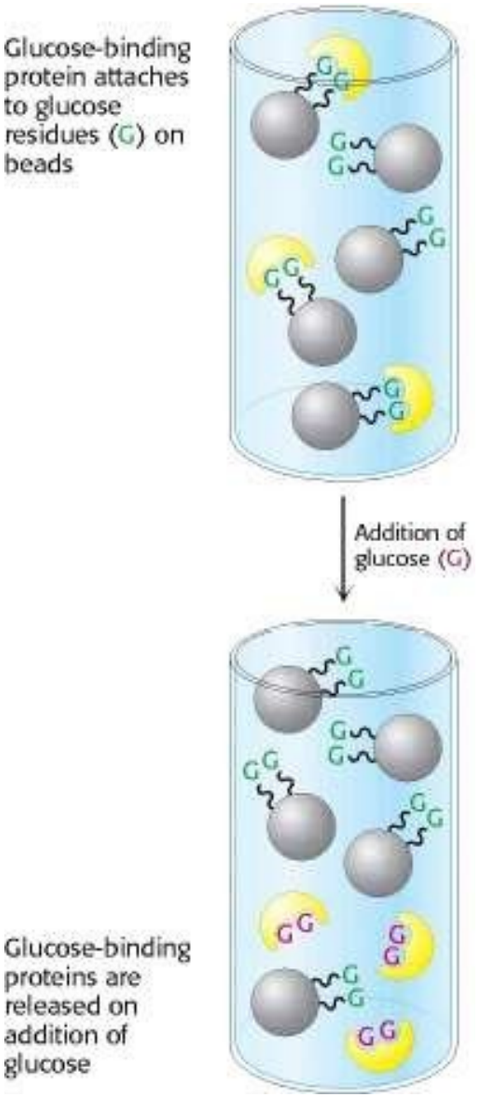
The bound concanavalin A can be eluted by adding a concentrated solution of glucose that displaces the column-attached glucose residues from binding sites on concanavalin A

▪ Affinity chromatography can be effectively used to isolate a protein that reconises group X by

✓(1) covalently attaching X or a derivative of it to a column

✓ (2) adding a mixture of protein to this column, which is then washed with buffer to remove unbound proteins

✓(3) eluting the desired protein by adding a high concentration of a soluble form of X or the condition to decrease binding affinity



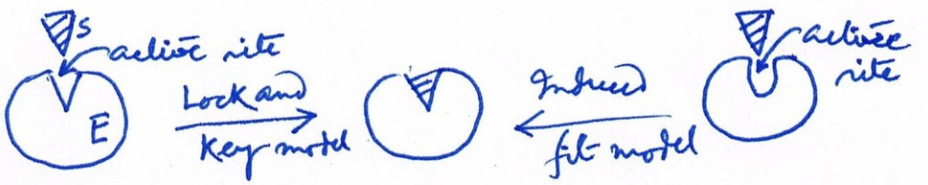
Enzyme Catalysis:

A catalyst is a substance that accelerates a reaction but undergoes no net chemical change. The catalyst lowers the activation energy of the reaction by providing an alternative path that avoids the slow, rate-determining step of the ~~uncat~~ uncatalyzed reaction.

Enzymes which are ~~homogeneous~~ homogeneous biological catalyst are very specific and have dramatic effect on the reaction rates.

Enzymes act in aqueous environment ~~of~~ of cells. These biologically ubiquitous compounds are proteins that contain active site, which is responsible for binding the substrates, the reactants and processing them into products. ~~Enzyme~~ The structure of the active site is specific to the reaction that it catalyzes, with groups in the substrate interacting with groups in the active site by the intermolecular interactions, such as H-bonding, electrostatic forces and van der Waals interaction.

(7)



Two models that explains the binding of a substrate to the active site of an enzyme. In the lock-and-key model, the active site and substrate have complementary three dimensional structure and dock without the need for major atomic rearrangement. In the induced fit model, binding of the substrate induces a conformational change in the active site. The substrate fits well in the active site after the conformational change has taken place. Experimental evidence favours the induced fit model.

Michaelis - Menten mechanism :

Enzyme kinetics are typically conducted by monitoring the initial rate of product formation in solution phase where enzyme is present at a very low concentration.

In Michaelis - Menten mechanism, an enzyme - substrate complex is formed in the first step and either the substrate is released unchanged or after modification products are formed.

$$E + S \underset{k_a'}{\overset{k_a}{\rightleftharpoons}} ES \xrightarrow{k_b} P + E.$$

'ES' is the bound state of the enzyme and its substrate.

The rate of formation of product is :

$$v = k_b [ES]$$

The net rate of formation of the enzyme - substrate complex is:

$$\frac{d}{dt}[ES] = k_a [E].[S] - k_a' [ES] - k_b [ES]$$

Steady - state approximation :

During the major part of a reaction, the concentration and the rate of change of all reaction intermediates are constant and small.

$$\therefore \frac{d[ES]}{dt} = k_a [E].[S] - k_a' [ES] - k_b [ES] = 0$$

$$\text{or} \quad [ES] = \frac{k_a [E].[S]}{k_a' + k_b}$$

$[E]$ and $[S]$ are the concentration of the free enzyme and free substrate

If $[E]_0$ is the total concentration of enzyme

$$[E] + [ES] = [E]_0$$

Since only a little enzyme is added and typically substrate is in large excess relative to enzyme, the free substrate concentration is approximately equal to the initial substrate concentration $[S] \approx [S]_{total}$

$$[ES] = \frac{k_a ([E]_0 - [ES])[S]}{k_a' + k_b}$$

$$\therefore \quad [ES] = \frac{K_a [E]_o [S]}{K_a' + K_b + K_a [S]}$$

∴ The rate of formation of product

$$v = \frac{d[P]}{dt} = K_b [ES]$$

$$= \frac{K_a K_b [E_o]_o [S]}{K_a' + K_b + K_a [S]}$$

$$= \frac{K_b [E]_o [S]}{\frac{K_a' + K_b}{K_a} + [S]}$$

$$\therefore \boxed{\frac{d[P]}{dt} = \frac{K_b [E]_o [S]}{K_M + [S]}} \quad \ldots (1)$$

$K_M = \frac{K_a' + K_b}{K_a}$ ← Michaelis constant

Eqn (1) ⇒ The rate of enzymolysis depends linearly on the concentration of enzyme $[E]_o$, but in a more complicated way on the concentration of substrate.

$K_M = \frac{(K_a' + K_b)}{K_a}$ is characteristic of a given enzyme acting on a given substrate and having the dimension of molar concentration.

when $[S] \gg K_M \Rightarrow K_M + [S] \approx [S]$

$$\frac{d[P]}{dt} = K_b [E]_o$$

The reaction is zeroth order in S. This means that the rate is constant because there is so much 'S' present that it remains at effectively the same concentration even though products are being formed ⇒ the rate of enzymolysis ~~reaches~~ & the rate of formation of product reaches its maximum value.

$v = v_{max} = K_b [E]_o$ ⇒ maximum velocity of the enzymolysis

when $[S] \ll K_M$, a little amount of S is present

$$K_M + [S] \approx K_M \Rightarrow \frac{d[P]}{dt} = \frac{K_b}{K_M}[E]_0[S]$$

The rate is proportional to $[S]$ as well as $[E]_0$.

$$\frac{d[P]}{dt} = \frac{K_b[S]}{K_M + [S]}[E]_0 = \frac{K_b[E]_0[S]}{K_M + [S]}$$

$$\text{or} \quad v = \frac{v_{max}}{\frac{K_M}{[S]} + 1}$$

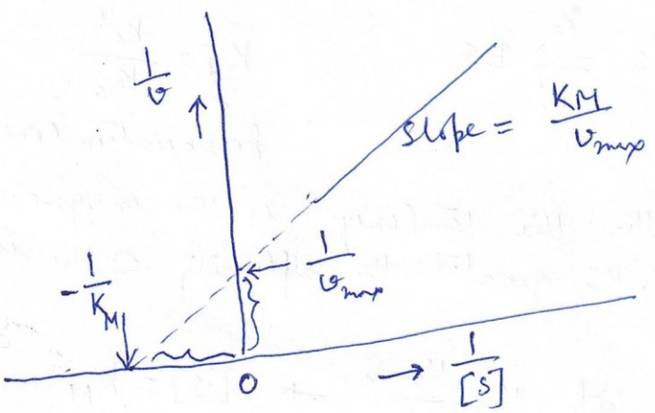
$$\text{or} \quad \frac{1}{v} = \frac{1}{v_{max}}\left(1 + \frac{K_M}{[S]}\right)$$

$$\therefore \frac{1}{v} = \frac{1}{v_{max}} + \left(\frac{K_M}{v_{max}}\right)\frac{1}{[S]}$$

A Lineweaver-Burk plot is a plot of $\frac{1}{v}$ against $\frac{1}{[S]}$.
(double reciprocal plot) $\Rightarrow$ A straight line with

$$\text{slope} = \frac{K_M}{v_{max}}, \text{ a 'y' intercept} = \frac{1}{v_{max}}$$

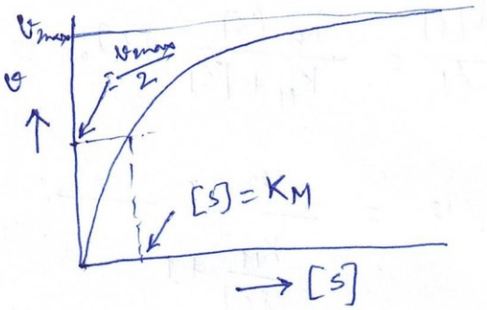
and 'x' intercept $= -\frac{1}{K_M}$



However, the plot cannot give the individual rate constants $K_2 \times K_2'$
Stopped-flow technique can give the additional data needed.

$$v = \frac{v_{max}}{1 + \frac{K_M}{[S]}}$$

or $$v = \frac{[S]\, v_{max}}{[S] + K_M}$$



Variation of the rate of an enzyme catalysed reaction with substrate conc.

if $[S] = K_M$

$$v = \frac{v_{max}}{2}$$

Thus Michaelis constant K_M is the substrate concentration at which v is half the maximum velocity ($v_{max}/2$) attainable at a particular concentration of enzyme.

The affinity of an enzyme for its substrate is the inverse of the dissociation constant K_d for the dissociation of the enzyme substrate complex ES.

$$E + S \underset{K_b'}{\overset{K_a}{\rightleftharpoons}} ES \qquad K_d = \frac{K_b'}{K_a}$$

↑ dissociation constant

⇒ the smaller the tendency of the enzyme and its substrate to dissociate, the greater the affinity of the enzyme for its substrate

at $v = \frac{v_{max}}{2} \rightarrow [S] = K_M$

$$K_M = \frac{K_b' + K_b}{K_a} = [S]$$

if $K_a' \gg K_b$

then $K_a' + K_b \simeq K_a'$

$$\therefore [S] = \frac{K_a' + K_b}{K_a} \approx \frac{K_a'}{K_a} = K_d$$

$\therefore K_M$ is approximately equal to K_d when the association and dissociation of the ES complex is rapid relative to the rate-limiting step in the enzymolysis

At large substrate concentration:

Michaelis-Menten Equation: $v = v_{max} = K_b[E]_0$

The velocity of an enzyme-catalysed reaction becomes independent of substrate concentration when enzyme is fully saturated with substrate in the steady state and when the concentration of free enzyme is negligible ⇒ The maximum velocity v_{max} is proportional to $[E]_0$; $v_{max} = K_{cat}[E]_0$ again $v_{max} = K_b[E]_0$; The turnover frequency or catalytic constant is the rate of breakdown of ES complex to products K_{cat} which is K_b

$$E + S \underset{K_a'}{\overset{K_a}{\rightleftharpoons}} ES \xrightarrow{K_b} P + E \qquad K_{cat} = K_b = \frac{v_{max}}{[E]_0}$$

At low substrate concentration:

Michaelis-Menten Equation $\quad v = \frac{K_b}{K_M}[E]_0[S] = \frac{K_{cat}}{K_M}[E]_0[S]$

Most of the enzyme is in free form.

$E + S \xrightarrow{K} ES$; $v = K_a[E][S]$; $\frac{K_{cat}}{K_M}$ represents the apparent rate constant for combination of a substrate with free enzyme. Because an enzyme and substrate can not combine more rapidly than diffusion permits, there is an upper limit on enzyme catalysis. The value of K_{cat}/K_M can not be greater than the diffusion limit ~~of enzyme catalysis~~ of about $10^9\ s^{-1}M^{-1}$. If K_{cat}/K_M approaches the diffusion limit then it indicates of enzyme efficiency in binding the substrate and converting it into products. ⇒ K_{cat}/K_M is defined as catalytic efficiency of an enzyme (η) $\quad \eta = K_{cat}/K_M \rightarrow$ upper limit $10^8 - 10^9\ s^{-1}M^{-1}$