SHRIMATI INDIRA GANDHI COLLEGE

(Nationally Accredited at 'A' Grade (3rd Cycle) By NAAC)
Tiruchirappalli – 2.

INSTRUCTION MATERIAL

2017-2018

DEPARTMENT OF BIOCHEMISTRY

CONTENT

Laboratory Manual for Food and Enzymes

Objectives:

1. To assay the activity of enzymes from different sources.
2. To stimulate their interest in learning the structure, function and kinetics of enzyme and their role as catalyst and regulator of cell metabolism and to serve as foundation for more advanced enzymology courses

Contents

1. Estimation of proteins by Lowry / Brad ford method
2. Estimation of phospholipids by phosphorous assay
3. Estimation of sodium and potassium by Flame photometry
4. Effect of pH, temperature and substrate concentration for amylase and urease and determination of Vmax & Km
5. Effect of inhibitor on activity of any one enzyme
6. Effect of activator on activity of any one enzyme
7. Desalting of proteins by dialysis
8. Separation of polar and non polar lipids by TLC
9. Rf value calculation of various amino acids using TLC and PC
10. Separation of serum proteins by paper electrophoresis

Estimation of Protein by Lowry's Method

Aim

To estimate the amount of protein present in the given sample by Folin Ciocalteau method.

Principle

This method is more sensitive than the biuret method since it determines the low concentration of protein. The reagent called Folin Ciocalteau is quite complex and contains phosphomolybdic acid and tungstate. The aromatic amino acid tyrosine and tryptophan present in protein reacts with these and produce a dark blue colour which can be read at 640nm (red filter).

Reagents Required

1**) Preparation of stock solution:**

200mg of BSA was weighed accurately and dissolved in 100ml of water.

2) **Working standard:**

10ml of stock solution was taken and diluted to 100ml with distilled water.

3**) Alkaline copper reagents:**

Solution A: 2% of $NaCO_3$ in 0.1 N NaOH.

Solution B: 0.5% of $CUSO_4$ in 1% sodium potassium tartarate solution.

Mix 50 ml of solution A and 1 ml of solution B just before use.

4) Folin Ciocalteau Reagent:

Into a 2 litre flask, measure out 100g of sodium tungstate, 25 g of sodium molybdate, 500ml distilled water, 50 ml of 85% phosphoric acid, 100ml of conc. HCL. The mixture is refluxed gently for about 10 hours with a condenser. After cooling, 150g of lithium sulphate, 50ml of distilled water and a few drops of bromine are added and boiling continued for another 10 minutes without the condenser. This helps to remove excess bromine. After cooling the volume is made up to 1000ml and filtered if necessary. The filtrate should not have any greenish tint. If it has, boil it with bromine once more. This stock reagent diluted with equal volume of water before use.

Alternatively ready to use refluxed Folin Ciocalteau reagent (2N) was diluted with the equal volume of distilled water just before use (2ml of commercial reagent +2ml of DW).

Preparation of Sample

5 gram of wheat flour was dissolved in 100 ml of water and filtered the filtrate was used as sample.

Procedure:

0.2ml to 1ml of working standard was pipetted out into different labeled tubes. The volume was made up to 1ml with water. To this 4.5ml of alkaline copper reagent was added, mixed well. Allow it to stand for 10 minutes at room temperature. 0.5ml of folins phenol reagents was added and allowed it to stand for 20 minutes at room temperature. For test 0.1ml of sample was taken and 1ml of water was taken as a blank. They were treated as standard. The blue color developed was read at 640nm in a colorimeter. The amount of protein is calculated from the graph.

Result:

The amount of Protein present in the given sample was ----------.

Estimation of Protein by Lowry's Method

S.NO	CONTENT	B	S1	S2	S3	S4	S5	T1	T2
1.	Volume of standard (ml)	---	0.2	0.4	0.6	0.8	1.0	---	---
2.	Concentration in µg	---	40	80	120	160	20 0	---	---
3.	Volume of test solution (ml)	---	---	---	---	---	---	0.1	0.1
4.	Volume of distilled Water (ml)	1	0.8	0.6	0.4	0.2	---	0.9	0.9
5.	Volume of alkaline copper reagent (ml)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Allow it to stand for 10 minutes at room temperature									
6.	Vol. of folin phenol reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Allow it to stand for 20 minutes at room temperature and blue color developed was read at 640nm.									
7.	Optical density at 640nm.								

Calculation:

----------O.D corresponds to ---------- µg

0.1ml of sample contains----------µg

100ml of sample contain = ×100/ 0.1 µg

= µg / 1000 = mg.

100ml of sample was made from 5gm of wheat flour

∴ 5g of wheat flour contain = mg

100g of wheat flour contain = ×100/5 mg

= mg / 1000 = g/dl.

Concentration:

200mg = 100ml

? = 10ml **10ml = 20mg**

100ml = 20ml

0.2ml = ? **0.2ml = 0.04mg = 0.04×1000 = 40µg.**

Estimation of Phospholipids

Aim

To estimate the amount of phospholipids present in the oil

Principle

Phosphate reacts with molybdic acid to form phosphomolybdic acid on treatment with amino napthol sulphonic acid (ANSA). Phosphomolybdic acid is reduced to produce a deep blue colour which is probably a mixture of oxides of molybdenum. The blue colour developed is read at 620nm.

Reagents Required

Stock solution

35.1 mg of Potassium di hydrogen phosphate in 100ml of water.

Working standard

10 ml of stock is diluted to 100 ml

Ammonium molybdate

2.5g of ammonium molybdate in 100 ml of 3N sulphuric acid.

ANSA

Add 0.5g of ANSA to 195 ml of 15% sodium bisulphite and 5 ml of 20% sodium suphite. Stir until it dissolves. Store it in brown bottle.

Test solution

1 ml of oil is treated with 5 drops of perchloric acid and heated in a boiling water bath to digest the mixture. The content in the tube should be just boiling. Initially the sample would get charred and latter get cleared. At this stage the digestion is complete. Allowed to cool and it is used as test solution.

Procedure

The standard in the range of 0.5 -2.5 ml was taken in a series of test tubes. 1ml of test sample was also taken in "T" test tube. All the test tubes were made up to 9 ml with distilled water. A blank containing 9 ml of water alone was also taken. To all the test tubes 1 ml of ammonium molybdate and 0.4 ml of ANSA were added. The blue colour developed was read at 620nm after 10 minutes.

Result

The amount of phospholipids present in the given sample was ----------.

Estimation of Phospholipids									
S.No	Content	B	S_1	S_2	S_3	S_4	S_5	T_1	T_2
1	Volume of standard solution (ml)	-	0.5	1	1.5	2	2.5		
2	Concentration of Solution (ìg)		4	8	12	16	20		
3	Volume of Test solution (ml)							1	1
4	Volume of Distilled water (ml)	9	8.5	8	7.5	7	6.5	8	8
5	Volume of ammonium molybdate (ml)	1	1	1	1	1	1	1	1
6	Volume of ANSA (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
The blue developed was read at 620 nm									
7	Optical density at 620 nm								

Calculation

---------- OD corresponds to ------ ìg of Phosphorus

∴ 1 ml of test solution contain ----- ìg of Phosphorus

1 ml of test solution was prepared from 1ml of oil

1ml of oil contain --- ìg of Phosphorus

∴ 100ml of oil contain = ----X100/1= ----- ìg of Phosphorus

Amount of Phospholipid = Amount of Phosphorus X 25

Amount of Phospholipid present in oil = ----- X25=---------- ìg of Phospholipid

i.e = ------ /1000=------ mg of Phospholipid

Estimation of Sodium and Potassium by Flame Photometry

Aim:

To estimate the amount of Sodium and Potassium using flame photometry

Principle

When a liquid sample containing a metallic salt solution is introduced into a flame, the solvent vaporizes leaving solid salt. The salt in turn is converted into gaseous state. All the parts of the gaseous molecule are dissociated to give free neutral atoms (or) radicals excited by thermal energy of the flame. The excited atoms which are unstable quickly emit photons and return to lower energy state. The measurement of the emitted photons forms the basis of flame photometry.

Instrumentation

It consists of a burner in which fuel LPG and air used under controlled conditions to produce a blue cone shaped flame. The nebulizer is used to aspirates the sample. The emission in the flame is measured using filters specific for sodium and potassium.

Preparation of Stock Standard

Sodium (Na) – 1000 mmol/litre

Dissolve 58.5 g of sodium chloride in 1 Litre of double distilled water.

Potassium (K) – 100 mmol/litre

Dissolve 7.46 g of potassium chloride in 1 litre of double distilled water.

Preparation of stock standards (Na-140/5 mmol/L)

Take 70 ml from sodium stock standard solution and add 25 ml from potassium stock standard solution. Then it is made up to 500 ml with double distilled water.

Preparation of working standard

Dilute the above solution to 1:100 with DDW

Sample preparation

Dilute the clear serum with double distilled water 1:100. Avoid unusual turbidity as this more value.

Connect all the connection correctly and lighten the flame in blue cones, run the double distilled water as blank by adjusting the digital display to 000. Run the standard for sodium by adjusting to 140. Repeat the blank reading to ensure 000. Again read standard sodium at 140.

Repeat for several times. Then simultaneously run with test solution without disturbing the adjustment and record the results. Change the filter, in the same way run for potassium, adjusts the standard for K to 5. Then simultaneously run the test solution without disturbing the adjustments and record the results.

Filter used for Na-Orange

Filter used for K- Deep red

Results

The amount of sodium in the given sample was ----------

The amount of potassium in the given sample was ----------

Effect of substrate concentration on salivary amylase activity

Aim:

To determine the effect of substrate concentration on the activity of salivary amylase.

Principle:

The starch forms a complex with iodine in acidic medium giving a blue colour which is read colorimatrically at 660nm upon treatment with enzyme hydrolysis of starch takes place, gradually losing its linkage which is used for the assessment of enzyme activity.

Reagent required:

Phosphate Buffer (pH-7)

Solution A:

17.8g of disodium hydrogen phosphate in 1litre of water (0.1M).

Solution B:

15.6g of sodium dihydrogen phosphate in 1litre of water (0.1M).

Mix 69ml of solution A and 31ml of solution B to form 100ml.

Buffered substrate:

Starch: phosphate Buffer (4:5)

100mg of starch was dissolved in 100ml of distilled water and warmed in a water bath to dissolve starch. 40ml of starch solution was taken and mixed with 50ml of phosphate buffer

Stock iodine solution:

3g of potassium iodide was dissolved in 50ml of water and added 1.3g of iodine. The solution was to be made up to 100ml with distilled water.

Working iodine solution:

10ml of iodine solution was made up to 100ml with distilled water.

Saline:

900mg of sodium chloride was dissolved in 100ml of water.

Enzyme:

1ml of saliva was made up to 10ml with saline.

Procedure:

Pipetted out various concentrations of buffered substrate into a series of test tubes and the volume was made up to 3 ml with water. 0.5 ml of enzyme was added to "T" test tubes alone. The test tubes were incubated at 37°C for 15 minutes. The reaction was stopped by the

addition of 0.4 ml of iodine. Then the enzyme was added to the control test tubes alone. The total volume was made up to 4.1ml with water in all the test tubes. The blank was prepared according to the table. The colour developed was read at 660nm.

Result:

The Km and V max for the effect of substrate concentration on the activity of salivary amylase by **Michaelis – Menten plot** was

Km =

V max =

The Km and V max for the effect of substrate concentration on the activity of salivary amylase by **Line weaver Burk plot** was

Km =

V max =

Effect of substrate concentration on salivary amylase activity

S.No	Content	B	C_1	T_1	C_2	T_2	C_3	T_3	C_4	T_4	C_5	T_5	C_6	T_6
1	Buffered Substrate (ml)	-	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.5	0.5	0.6	0.6
2	Water (ml)	3	2.9	2.9	2.8	2.8	2.7	2.7	2.6	2.6	2.5	2.5	2.4	2.4
3	Enzyme (ml)	-	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5
Incubated at 37°C for 15 minutes														
4	Iodine (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
5	Enzyme (ml)	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-
6	Water (ml)	4.6	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1
7	OD at 660 nm													
8	OD difference													

Model calculation

Line weaver Burk plot

Let the difference in OD were 0.02, 0.04, 0.06, 0.08, 0.08, 0.08

[S] µg	1/ [S]	V	1/V
250	$0.004=4x10^{-3}$	0.02	50
500	$0.002=2x10^{-3}$	0.04	25
750	$0.0013=1.3x10^{-3}$	0.06	16.67
1000	$0.001=1x10^{-3}$	0.08	12.5
1250	$0.0008=0.8x10^{-3}$	0.08	12.5
1500	$0.000667=0.67x10^{-3}$	0.08	12.5

From the graph

$-1/km= -0.04x10^{-3}$

$\therefore$ $km= 1/0.04x10^{-3} = 1/40=0.025\mu g$

Effect of pH on salivary amylase activity

Aim:

To determine the effect of pH on salivary amylase activity.

Principle:

The starch forms a complex with iodine in acidic medium giving a blue colour which is read colorimatrically at 660nm upon treatment with enzyme hydrolysis of starch takes place, gradually losing its linkage which is used for the assessment of enzyme activity.

Reagent required:

Phosphate Buffer (pH - 6-7.6)

Solution A:

13.8g of sodium dihydrogen phosphate anhydrous in 1litre of water (0.1M).

Solution B:

26.8 g of disodium hydrogen phosphate .7H_2O in 1litre of water (0.1M).

Buffered substrate:

Starch: phosphate Buffer (4:5)

100mg of starch was dissolved in 100ml of distilled water and warmed in a water bath to dissolve starch. 40ml of starch solution was taken and mixed with 50ml of phosphate buffer

Stock iodine solution:

3g of potassium iodide was dissolved in 50ml of water and added 1.3g of iodine. The solution was to be made upto 100ml with distilled water.

Working iodine solution:

10ml of iodine solution was made upto 100ml with distilled water.

Saline:

900mg of sodium chloride was dissolved in 100ml of water.

Enzyme Source:

1ml of saliva was made up to 10ml with saline.

Phosphate buffer at various pH were prepared as follows (pH – 5.8-7.8)

pH	Solution A (ml)	Solution B (ml)	water in ml
5.8	92	8	100
6	87	13	100
6.2	81.5	18.5	100
6.4	73.5	26.5	100
6.6	62.5	37.5	100
6.8	51	49	100
7	39	61	100
7.2	28	72	100
7.4	19	81	100
7.6	13	87	100
7.8	8.5	91.5	100

Procedure:

Pipetted out 1ml of buffered substrate of different pH (6, 6.4, 6.8, 7.2, 7.6) into a series of test tubes. 0.5 ml of enzyme was added to "T" test tubes alone. The test tubes were incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 0.4 ml of iodine. Then the enzyme was added to the control test tubes alone. The total volume was made up to 7.1 ml with water in all the test tubes. The blank consisted of 0.4ml of iodine and 7.6ml of water. The blue colour developed was read at 660nm.

Result:

The optimum pH of salivary amylase at 37°C was 6.8

Effect of pH on salivary amylase activity

S.No	Content	B	pH - 6.0		pH - 6.4		pH - 6.8		pH - 7.2		pH -7.6	
			C_1	T_1	C_2	T_2	C_3	T_3	C_4	T_4	C_5	T_5
1	Buffered Substrate (ml)	-	1	1	1	1	1	1	1	1	1	1
2	Enzyme (ml)	-	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5
Incubated at 37°C for 15 minutes												
3	Iodine (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
4	Enzyme (ml)	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-
5	Water (ml)	7.6	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1
6	OD at 660 nm											
7	OD difference											

Effect of temperature on salivary amylase activity

Aim:

To determine the effect of temperature on salivary amylase activity.

Principle:

The starch forms a complex with iodine in acidic medium giving a blue colour which is read colorimatrically at 660nm upon treatment with enzyme hydrolysis of starch takes place, gradually losing its linkage which is used for the assessment of enzyme activity.

Reagent required:

Phosphate Buffer (pH-7)

Solution A:

17.8g of disodium hydrogen phosphate in 1litre of water (0.1M).

Solution B:

15.6g of sodium dihydrogen phosphate in 1litre of water (0.1M).

Mix 69ml of solution A and 31ml of solution B to form 100ml.

Buffered substrate:

Starch: phosphate Buffer (4:5)

100mg of starch was dissolved in 100ml of distilled water and warmed in a water bath to dissolve starch. 40ml of starch solution was taken and mixed with 50ml of phosphate buffer.

Stock iodine solution:

3g of potassium iodide was dissolved in 50ml of water and added 1.3g of iodine. The solution was to be made upto 100ml with distilled water.

Working iodine solution:

10ml of iodine solution was made upto 100ml with distilled water.

Saline:

900mg of sodium chloride was dissolved in 100ml of water.

Enzyme Source:

1ml of saliva was made up to 10ml with saline.

Procedure:

Pipetted out 1ml of buffered substrate was added into a series of test tubes. 0.5 ml of enzyme was added to "T" test tubes alone. The test tubes were incubated at different temperatures (15°C, 25°C, 37°C, 45°C, and 55°C) for 15 minutes. The reaction was stopped

by the addition of 0.4 ml of iodine. Then the enzyme was added to the control test tubes alone. The total volume was made up to 7.1 ml with water in all the test tubes. The blank consisted of 0.4ml of iodine and 7.6ml of water. The blue colour developed was read at 660nm.

Result:

The optimum temperature of salivary amylase was 37°C

Effect of temperature on salivary amylase activity

S.No	Content	B	15°C		25°C		37°C		45°C		55°C	
			C_1	T_1	C_2	T_2	C_3	T_3	C_4	T_4	C_5	T_5
1	Buffered Substrate (ml)	-	1	1	1	1	1	1	1	1	1	1
2	Enzyme (ml)	-	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5
Incubated at various temperatures for 15 minutes												
3	Iodine (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
4	Enzyme (ml)	-	0.5	-	0.5	-	0.5	-	0.5	-	0.5	-
5	Water (ml)	7.6	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1
6	OD at 660 nm											
7	OD difference											

Effect of substrate concentration on Urease activity

Aim

To study the effect of substrate concentration on urease activity.

Principle

Depending on the affinity of the enzyme towards the substrate, K_m value changes. Thus greater the affinity of the enzyme for the substrate the lesser will be the K_m value. Lesser the affinity of the enzyme for the given substrate higher will be the K_m value. By applying MM equation and Line weaver burk plot it is possible to determine the K_m and V_{max} of any substrate. When Urea solution is incubated with urease, which converts urea into carbon dioxide and ammonia. Nesslers reacts with ammonia to give yellow colour which is read colourimetrically at 520 nm.

Reagents Required

Enzyme source

Soak 5g of powdered horse gram in 100 ml of water. Leave it for half an hour and filtered and the filtrate was used as enzyme source.

Phosphate Buffer (pH - 7)

Solution A:

13.8g of sodium dihydrogen phosphate anhydrous in 1litre of water (0.1M).

Solution B:

26.8 g of disodium hydrogen phosphate .$7H_2O$ in 1litre of water (0.1M).

Phosphate buffer at pH – 7.0

pH	Solution A (ml)	Solution B (ml)	water (ml)
7	39	61	100

Buffered substrate of varying concentration (0.1- 0.5%)

Prepare varying concentration (0.1- 0.5%) of urea in phosphate buffer at pH - 7

1N Suphuric acid as enzyme inhibitor (100 ml)

$N_1 V_1 = N_2 V_2$

$36 \times ? = 1X100$

$\therefore X=1X100/36$

10% sodium tungstate

10 g in 100 ml of DW

Nessler's reagent

Dissolve 100 g mercuric iodide, 40g of potassium iodide in 400ml of DW. Dissolve 100g of Sodium hydroxide to the iodine solution with stirring. Make up this volume 1000ml and filter it.

Procedure

Into a series of test tubes pipetted out 1ml of buffered substrate of varying concentration (0.1 – 0.5%) as "Controls" and "Tests" labeled test tubes. The tubes were incubated at 60°C for 2 minutes for acclimatization. Add 1ml of 1N sulphuric acid to all the "C" tubes alone as enzyme inhibitor. Add 1ml of enzyme to all the tubes and incubate at 60°C for 15 minutes. After incubation add 1ml of 1N sulphuric acid to all the "T" tubes alone as enzyme inhibitor. Then add 1 ml of 10% sodium tungstate to all the tubes to precipitate the protein. Allowed to stand for 5 minutes and centrifuged. Take 0.5 ml of supernatant and made upto 9.5 ml with distilled water. Add 0.5ml of nessler's reagent and the yellow colour developed was read at 520 nm. Plot the graph and calculate the K_m and V_{max} by MM equation and by LB plot

Result

The Km and V max for the effect of substrate concentration on the activity of urease by **Michaelis – Menten plot** was

K_m =

V_{max} =

The Km and V max for the effect of substrate concentration on the activity of urease by **Line weaver Burk plot** was

K_m =

V_{max} =

Effect of substrate concentration on Urease activity

S.No	Contents	B	C_1	T_1	C_2	T_2	C_3	T_3	C_4	T_4	C_5	T_5
1	Buffered Substrate (ml)	-	2.5	2.5	2	2	1.5	1.5	1	1	0.5	0.5
Incubated at 60°C for 2 minutes for acclimatization												
2	Volume of sulphuric acid (ml)	-	1	-	1	-	1	-	1	-	1	-
3	Enzyme (ml)	-	1	1	1	1	1	1	1	1	1	1
Incubated at 60°C for 15 minutes												
4	Volume of sulphuric acid (ml)	-	-	1	-	1	-	1	-	1	-	1
5	Volume of sodium tungstate (ml)	-	1	1	1	1	1	1	1	1	1	1
Allowed to stand for 5 minutes and centrifuged												
6	Supernatant (ml)	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
7	Distilled water (ml)	9.5	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
8	Nessler's Reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
9	OD at 520 nm											
10	OD difference											

Effect of pH on Urease activity

Aim

To study the effect of pH on urease activity.

Principle

Drastic changes in pH modify the activity of enzyme due to ionization and deionization of active site. When Urea solution is incubated with urease, which converts urea into carbon dioxide and ammonia. Nesslers reacts with ammonia to give yellow colour which is read colourimetrically at 520 nm.

Reagents Required

Enzyme source

Soak 5g of powdered horse gram in 100 ml of water. Leave it for half an hour and filtered and the filtrate was used as enzyme source.

Phosphate Buffer (pH - 6-7.6)

Solution A:

13.8g of sodium dihydrogen phosphate anhydrous in 1litre of water (0.1M).

Solution B:

26.8 g of disodium hydrogen phosphate .7H_2O in 1litre of water (0.1M).

Phosphate buffer at various pH were prepared as follows (pH – 6-7.6)

H	Solution A (ml)	Solution B (ml)	Water (ml)
5.8	92	8	100
6	87	13	100
6.2	81.5	18.5	100
6.4	73.5	26.5	100
6.6	62.5	37.5	100
6.8	51	49	100
7	39	61	100
7.2	28	72	100
7.4	19	81	100
7.6	13	87	100
7.8	8.5	91.5	100

Buffered substrate

Prepare 1% urea in phosphate buffer of varying pH

1N Suphuric acid as enzyme inhibitor (100 ml)

$N_1 V_1 = N_2 V_2$

36x? = 1X100

∴ X=1X100/36

10% sodium tungstate

10 g in 100 ml of DW

Nessler's reagent

Dissolve 100 g mercuric iodide, 40g of potassium iodide in 400ml of DW. Dissolve 100 g of Sodium hydroxide to the iodine solution with stirring. Make up this volume 1000ml and filter it.

Procedure

Pipetted out 4ml of buffered substrate of different pH (6.4, 6.8,7, 7.4, 7.8) into a series of test tubes. Add 1ml of 1N sulphuric acid to all the "C" tubes alone as enzyme inhibitor. Keep the tubes for inhibition for 2 minutes at 60°C. 1 ml of enzyme was added to all the test tubes and incubate at 60°C for 15 minutes. Add 1ml of 1N sulphuric acid to all the "T" tubes alone as enzyme inhibitor. Add 1 ml of 10% sodium tungstate to all the tubes to precipitate the protein. Allowed to stand for 5 minutes and centrifuged. Add 0.5ml of supernatant to 9 ml of water and 0.5ml of nessler's reagent. The blank consisted of 0.5ml of nessler's reagent and 9.5ml of water. The yellow colour developed was read at 520nm.

Result

The optimum pH for the activity of urease was found to be 7.

Effect of pH on Urease activity

S.No	Content	B	pH - 6.4		pH - 6.8		pH – 7.0		pH - 7.4		pH -7.8	
			C_1	T_1	C_2	T_2	C_3	T_3	C_4	T_4	C_5	T_5
1	Buffered Substrate (ml)	-	4	4	4	4	4	4	4	4	4	4
2	Volume of sulphuric acid (ml)	-	1	-	1	-	1	-	1	-	1	-
Incubated at 60°C for 2 minutes												
3	Enzyme (ml)	-	1	1	1	1	1	1	1	1	1	1
Incubated at 60°C for 15 minutes												
4	Volume of sulphuric acid (ml)	-	-	1	-	1	-	1	-	1	-	1
5	Volume of sodium tungstate (ml)		1	1	1	1	1	1	1	1	1	1
Allowed to stand for 5 minutes and centrifuged												
6	Supernatant (ml)	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
7	Distilled water (ml)	9.5	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
8	Nessler's Reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
9	OD at 520 nm											
10	OD difference											

Effect of Temperature on Urease activity

Aim

To study the effect of temperature on urease activity.

Principle

The velocity of the enzyme catalyzed reactions increases with increase in temperature. The temperature provides energy for the reaction. At optimum temperature the enzyme has maximum activity. But above optimum temperature the activity slows down and the reaction stops because the enzyme gets denatured. When Urea solution is incubated with urease, which converts urea into carbon dioxide and ammonia. Nesslers reacts with ammonia to give yellow colour which is read colourimetrically at 520 nm.

Reagents Required

Enzyme source

Soak 5g of powdered horse gram in 100 ml of water. Leave it for half an hour and filtered and the filtrate was used as enzyme source.

Phosphate Buffer (pH - 7)

Solution A:

13.8g of sodium dihydrogen phosphate anhydrous in 1litre of water (0.1M).

Solution B:

26.8 g of disodium hydrogen phosphate .$7H_2O$ in 1litre of water (0.1M).

Phosphate buffer at pH – 7.0

pH	Solution A (ml)	Solution B (ml)	water (ml)
7	39	61	100

Buffered substrate

Prepare 1% urea in phosphate buffer at pH - 7

1N Suphuric acid as enzyme inhibitor (100 ml)

$N_1 V_1 = N_2 V_2$

36x? = 1X100

$\therefore X=1X100/36$

10% sodium tungstate

10 g in 100 ml of DW

Nessler's reagent

Effect of Temperature on Urease activity

S.No	Content	B	30°C		45°C		60°C		75°C		90°C	
			C_1	T_1	C_2	T_2	C_3	T_3	C_4	T_4	C_5	T_5
1	Buffered Substrate (ml)	-	1	1	1	1	1	1	1	1	1	1
Incubated at 60°C for 2 minutes for acclimatization												
	Volume of sulphuric acid (ml)	-	1	-	1	-	1	-	1	-	1	-
3	Enzyme (ml)	-	1	1	1	1	1	1	1	1	1	1
Incubated at varying temperature for 15 minutes												
4	Volume of sulphuric acid (ml)	-	-	1	-	1	-	1	-	1	-	1
5	Volume of sodium tungstate (ml)	-	1	1	1	1	1	1	1	1	1	1
Allowed to stand for 5 minutes and centrifuged												
6	Supernatant (ml)	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
7	Distilled water (ml)	9.5	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
8	Nessler's Reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
9	OD at 520 nm											
10	OD difference											

Dissolve 100 g mercuric iodide, 40g of potassium iodide in 400ml of DW. Dissolve 100 g of Sodium hydroxide to the iodine solution with stirring. Make up this volume 1000ml and filter it.

Procedure

Into a series of test tubes pipetted out 1ml of buffered substrate as "Controls" and "Tests" labeled test tubes. The tubes were incubated at varying temperature (30°C, 45°C, 60°C, 75°C, 90°C) for 2 minutes. Add 1ml of 1N sulphuric acid to all the "C" tubes alone as enzyme inhibitor. Add 1ml of enzyme to all the tubes and incubate at varying temperatures

for 15 minutes. After incubation add 1ml of 1N sulphuric acid to all the "T" tubes alone as enzyme inhibitor. Then add 1 ml of 10% sodium tungstate to all the tubes to precipitate the protein. Allowed to stand for 5 minutes and centrifuged. Take 0.5 ml of supernatant and made upto 9.5 ml with distilled water. Add 0.5ml of nessler's reagent and the yellow colour developed was read at 520 nm.

Result

The optimum temperature for the activity of urease was found to be 60°C.

Effect of Inhibitor on Lactate Dehydrogenase activity

Aim:

To determine the effect of Inhibitor on Lactate Dehydrogenase activity.

Principle:

The enzyme activity assessed according to the method described by king (1965) with slight modification. Enzyme catalyzes the following the reaction

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightleftharpoons{\text{LDH}} NAD^{+} + \text{Lactate}$$

Lactate dehydrogenase acts on pyruvate to give lactate. During this reaction the reducing equivalent ($NADH+H^{+}$) gets oxidized. The yellow colour developed is read at 440 nm.

Reagent required:

1. **Enzyme source:**

   Added 2 ml of heparin to 5ml of blood. The mixture was allowed to stand for 5 minutes. The reaction mixture was centrifuged for 10 minutes. The supernatant was taken for assay as enzyme source.

2. **Buffered substrate:**

   Glycine Buffer: 0.1 m glycine and 0.1m NaCl in 1L of distilled water

   Substrate (Sodium pyruvate): 11 mg of sodium pyruvate is mixed with 50ml of glycine buffer.

3. **Dinitro phenyl hydrazine (DNPH) reagent**

   100 mg of DNPH is dissolved in 100 ml of 2N HCL

4. **Inhibitor (Acetone)**

   1 ml of acetone is made upto 50 ml of water.

5. **Co-factor**

   50 mg of NADH is dissolved in 100 ml of water.

6. **0.4N sodium hydroxide**

   8 g of NaOH is dissolved in 500 ml of distilled water.

Procedure:

Into a series of test tubes pipetted out 1 ml of buffered substrate and 0.1ml of enzyme to all the test tubes. 0.1ml was added to the "C" test tubes alone and all the test tubes were incubated in the water bath at 37°C. 0.2 ml of NADH was added to "T" test tubes alone. Increasing concentrations of inhibitor was added to all the test tubes and the test tubes were incubated at 37°C for 15 minutes.

1ml of DNPH was added to all the test tubes and again incubated at 37°C for 15 minutes. Then add 10ml of 0.4N NaOH with respect to inhibitor volume. A blank containing 1.2ml of water was treated same as control as test.

The yellow colour developed was read at 440 nm. A graph was plotted for OD values. From the graph the effect of inhibitor activity on LDH was determined.

Result

From the graph, increase in inhibitor concentration is found to decrease the activity of Lactate dehydrogenase proportionally.

Effect of Inhibitor on Lactate Dehydrogenase activity

S.No	Contents	B	C_1	T_1	C_2	T_2	C_3	T_3	C_4	T_4	C_5	T_5
1	Buffered Substrate (ml)	-	1	1	1	1	1	1	1	1	1	1
2	Enzyme (ml)	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
3	Volume of Distilled water (mL)	-	0.1	-	0.1	-	0.1	-	0.1	-	0.1	-
Incubated at 37°C for 15 minutes												
4	Volume of NADH	-	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
5	Volume of inhibitor (Acetone)	-	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.5	0.5
Incubated at 37°C for 15 minutes												
4	Volume of DNPH	1	1	1	1	1	1	1	1	1	1	1
5	Volume of NaOH (ml)	10	9.9	9.9	9.8	9.8	9.7	9.7	9.6	9.6	9.5	9.5
9	OD at 440 nm											
10	OD difference											

Effect of Activator on Lactate Dehydrogenase activity

Aim:

To determine the effect of activator on Lactate Dehydrogenase activity.

Principle:

The enzyme activity assessed according to the method described by king (1965) with slight modification. The enzyme catalyses the following reaction

$$\text{Puruvate} + \text{NADH} + \text{H}^{+} \overset{\text{LDH}}{\rightleftharpoons} \text{NAD}^{+} + \text{Lactate}$$

Lactate dehydrogenase acts on pyruvate to give lactate. During this reaction the reducing equivalent ($NADH+H^{+}$) gets oxidized. The yellow colour developed is read at 440 nm

Reagent required:

1. **Enzyme source:**

   Added 2 ml of heparin to 5ml of blood. The mixture was allowed to stand for 5 minutes. The reaction mixture was centrifuged for 10 minutes. The supernatant was taken for assay as enzyme source.

2. **Buffered substrate:**

   Glycine Buffer : 0.1 m glycine and 0.1m NaCl in 1L of distilled water

   Substrate (Sodium pyruvate): 11 mg of sodium pyruvate is mixed with 50ml of glycine buffer.

3. **Dinitro phenyl hydrazine (DNPH) reagent**

   100 mg of DNPH is dissolved in 100 ml of 2N HCL

4. **Activator (Magnesium sulphate)**

   1 g of Magnesium sulphate is dissolved in 50 ml of water.

5. **Co-factor**

   50 mg of NADH is dissolved in 100 ml of water.

6. **0.4N sodium hydroxide**

   8 g of NaoH is dissolved in 500 ml of distilled water.

Procedure:

Into a series of test tubes pipetted out 1ml of buffered substrate and 0.2ml of enzyme to all the test tubes. 0.2ml was added to the "C" test tubes alone and all the test tubes were incubated in the water bath at 37°C. 0.2 ml of NADH was added to "T" test tubes alone. Increasing concentrations of activator was added to all the test tubes and the test tubes were incubated at 37°C for 15 minutes.

1ml of DNPH was added to all the test tubes and again incubated at 37°C for 15 minutes. Then add 10ml of 0.4N NaOH with respect to activator volume. A blank containing 1.2ml of water was treated same as control as test.

The yellow colour developed was read at 440 nm. A graph was plotted for OD values. From the graph the effect of activator activity on LDH was determined.

Result

From the graph, increase in activator concentration is found to increase the activity of Lactate dehydrogenase proportionally.

Effect of Activator on Lactate Dehydrogenase activity

S.No	Contents	B	C_1	T_1	C_2	T_2	C_3	T_3	C_4	T_4	C_5	T_5
1	Buffered Substrate (ml)	-	1	1	1	1	1	1	1	1	1	1
2	Enzyme (ml)	-	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
3	Volume of Distilled water (mL)	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-
Incubated at 37°C for 15 minutes												
4	Volume of NADH	-	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
5	Volume of Activator [Magnesium sulphate]	-	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.5	0.5
Incubated at 37°C for 15 minutes												
4	Volume of DNPH	1	1	1	1	1	1	1	1	1	1	1
5	Volume of NaOH (ml)	10	9.9	9.9	9.8	9.8	9.7	9.7	9.6	9.6	9.5	9.5
9	OD at 440 nm											
10	OD difference											

Desalting of proteins by Dialysis

Aim:

To purify and desalt protein by dialysis.

Principle:

Dialysis is a process used to selectively remove small molecules from a sample containing mixture of both small and large molecules. It is commonly used for removing salt from protein. Dialysis is effectively achieved using a semipermeable membrane which allows only the small molecules to pass freely through the membrane and not the large colloidal protein molecules.

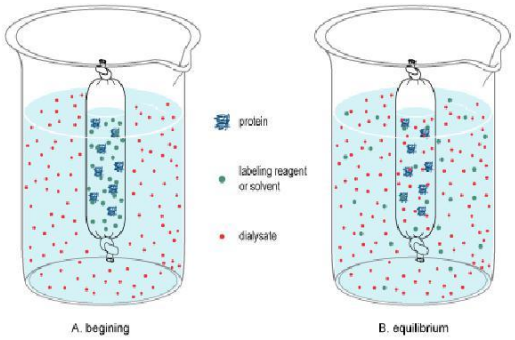
Material required:

Dialysis membrane

Gelatin

0.1N HCl

Thymol blue: 0.1g in 100ml of ethanol (filter if needed).



Procedure:

Preparation of the membrane:

Cut dialysis membrane into required size. The dry dialysis membrane contains 10% glycerol which can be removed by soaking in water for 10 minutes.

Desalting of protein

Take 50ml 5% gelatin into a 100ml beaker and 50ml of DW into another beaker. Added 1ml of thymol blue indicator into each of these beakers. To water added continuously drop by drop 0.1N HCl. This is done until the indicator had a colour between yellow-pink (pH 1.2-2.8) and red orange. Add 0.1N HCl until a similar shade is obtained in gelatin solution. The two solutions then had the same hydrogen ion concentration. Then transfer the gelatin solution into a dialyzing bag and suspend it into the beaker containing water for 4 hrs at room temperature.

Result

The gelatin solution gradually turned into yellow (decreased acidity). The outside solution turned pink (increased acidity) indicating the diffusion of hydrogen ion and small molecules through the membrane.

Separation of lipids by Thin layer chromatography

Aim

To separate the mixture of lipids by thin layer chromatography

Principle

Thin layer chromatography consists of thin layer adsorbent such as silica gel, alumina or cellulose on a glass plate or aluminium foil. This layer of adsorbent acts as a stationary phase. The adsorbent like silica has hydroxyl groups which act as interacting groups. The sample partitions between the mobile and the stationary phase, will move to various distance based on charge solubility and adsorption. The retention factor or Rf is the characteristics of the substance. The Rf value is the distance moved by the compound to that moved by the solvent.

Requirements

1. Thin layer chromatography plate 20X20cm
2. Silica gel
3. Thin layer chromatography apparatus
4. Petroleum ether-diethyl ether – acetone (90:10:1)/
   Toluene –ethyl acetate – formic acid (6:3:1)

Procedure

The plates were coated with slurry of silica gel in 0.02m sodium acetate to a thickness of 250µm. After spreading the plates were activated by heating at 105°C for 15 minutes. 5µl sample of various lipids were spotted as in paper chromatography. The plates were developed in petroleum ether-diethyl ether–acetone (90:10:1). The chamber was saturated with the same solvent system. The plates was then kept in the chamber and allowed to run until the solvent front rises to 3/4th of the plate.

The plate was removed, dried and solvent front was marked. From this value lipid sample were calculated. Iodine vapour is extensively used as a universal reagent for organic compounds. The iodine spot disappears rapidly but can be made more permanent by spraying 0.5% benzidine solution in absolute ethanol. Iodine vapour is concentrated in the form of cloud over the region where the compounds have separated.

Result

The given lipid was identified as ----------

Separation of amino acids by thin layer chromatography

Aim

To separate the mixture of amino acids by thin layer chromatography

Principle

Thin layer chromatography consists of thin layer adsorbent such as silica gel, alumina or cellulose on a glass plate or aluminium foil. This layer of adsorbent acts as a stationary phase. The adsorbent like silica has hydroxyl groups which act as interacting groups. The sample partitions between the mobile and the stationary phase, will move to various distance based on charge solubility and adsorption. The retention factor or Rf is the characteristics of the substance. The Rf value is the distance moved by the compound to that moved by the solvent.

Requirements

1. Thin layer chromatography plate 20X20cm
2. Silica gel
3. Thin layer chromatography apparatus
4. N-butanol-acetic acid-water (80:20:20).
5. Ninhydrin in acetone

Procedure

The plates were coated with slurry of silica gel in 0.02m sodium acetate to a thickness of 250µm. After spreading the plates were activated by heating at 105°C for 15 minutes. 5µl sample of various amino acids were spotted as in paper chromatography. The plates were developed in N-butanol-acetic acid-water (80:20:20). The chamber was saturated with the same solvent system. The plates was then kept in the chamber and allowed to run until the solvent front rises to 3/4th of the plate.

The plate was then removed; dried and solvent front was marked. The plates were sprayed with 0.3% solution of ninhydrin in acetone. The coloured spots developed by heating the plates at 110□C for 10 minutes. Once the colours were developed plates were exposed to vapours of concentrated ammonium hydroxide which helps in stabilization of colours.

Result

The given amino acids identified as ----------

Separation of Amino Acids by Paper Chromatography

Aim:

To separate and determine the RF value of amino acids by paper chromatography.

Principle:

Chromatography is a method by which a mixture of compounds in small quantities can be separated qualitatively. There are two phases, the stationary and mobile phase. When the mobile phase moves along the stationary phase separation of substance takes place. In paper chromatography the paper acts as an inert supporting media and the substances are applied as a spot on the paper and substance are carried along with the solvent as the partition co-efficient differs for different amino acids. Therefore each amino acid are separated individually.

Material Required

Whatmann No.1 circular chromatography paper.

Solvent system: n- butanol: glacial acetic acid: water (4:1:5 v/v)

Ninhydrin (Location reagent): 0.2% in acetone.

Procedure:

Whatmann No.1 Circular chromatography paper was taken. From the center a circle was drawn with a radius of 1 cm. At equal distances positions were marked for the application of sample. Each amino acid solution was applied separately on each spot by using a capillary tube. On the last spot the unknown amino acid was applied. The size of the spot should be as small as possible. The spots were air dried before development. The chamber was completely saturated with aqueous phase in order to obtain good separation. Into a petriplate was taken organic butanolic phase, the paper was kept in the petriplate, the solvent raised up the paper with the help of paper wick by capillary action. This arrangement was kept undisturbed for 30 minutes. The solvent front moved in a circular (radial) fashion. When it reached $3/4^{th}$ of the paper the run was stopped and the paper was removed the solvent front was marked and the paper was dried. Then 0.2% ninhydrin was sprayed on paper using sprayer. The paper was then dried for 10 minutes at 105 C. The amino acids appear as purple spots on white background and they were marked. The Rf was calculated by

$$RF = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent front}}$$

Result: The given unknown amino acids was identified as ----------

Separation of serum proteins by paper electrophoresis (cellulose acetate)

Aim:

To separate serum proteins by using paper electrophoresis.

Principle:

The principle is based on the migration of charged particle of any size in liquid medium under the influence of an electric field. Depending on kind of charge the molecule carry they move towards either to cathode (or) to an anode. An ampholyte become positively charged in acidic condition and migrate to cathode, in alkaline condition they become negatively charged and migrate to anode. The rate of migration of an ion in electric field depends on net charge of molecule, size and shape of particle.

Reagents required

Buffer – pH- 8.3 - 8.6

Tris – 600mg (50 mM Tris)

Glycine – 750mg (100 mM)

DW – 100ml

Cellulose actate strip – 1 No

Strip holding frame – 1

Ponceau S Stain (0.2 % Ponceau S in 3% TCA)

Ponceau S – 50 mg

TCA – 750 mg

DW – 25 ml

Destaining Solution (5% V/V acetic Acid, 7% V/V ethanol)

To prepare 50 ml

Acetic acid – 2.5 ml

Ethanol – 3.5 ml

DW 44 ml

Sample preparation

Take a drop of serum on a slide and add a few crystals of bromophenol blue and mix.

Procedure

Mark the cellulose paper strip +ve and –ve ends with pencil. At 1 cm away from the –ve end, mark the sample application line. Take about 10 ml of buffer in a petridish and place the cellulose acetate paper strip slowly using forceps. Allow 5-10 minutes for wetting. Place the wet cellulose acetate strip on the tissue paper and gently blot it. Apply the prepared sample on the sample application line. Place the cellulose strip on immediately onto the frame. Wet the whatmann filter paper wick in the buffer and blot it. Place the wick on the cellulose acetate strip at each end. Pour 25 ml of buffer to anode and cathode reservoir slowly. Close the lid and connect the tank to the power pack and switch on. Set the voltage knob to 100v and turn on the switch on the power pack. Continue the run for about 1 hour till the bromophenol blue dye reaches the +ve end. Switch off the power pack. Air dry the strips for about 10 minutes till the strip become completely dry. Place the strip in a petridish and add about 20 ml of Ponceau S Stain. Gently stain for in10 minutes. Destain for 5 minutes by shaking intermittently. Repeat few times with fresh destaining solution. Till the cellulose acetate background becomes white. Remove the strip and dry. Protein bands appear as pink bands.

The albumin moves most rapidly and so is found at the greatest distance from the start line. It is followed in turn by the á1, á2, â and ã globulins. The ã globulin has the smallest –ve charge. Fibrinogen (when plasma is used) is found as a narrow band between the â and ã globulin.

Result

In paper electrophoresis the serum can be separated into a number of fraction viz albumin, globulin (á1,á2, â, and ã) depending on sensitivity of the method of â can further be resolved into â1 and â2.

Immunology

Immunology

UNIT I –Immune System

Introduction

Immunology

The branch of biomedical science concerned with immunity. It is resistance of living organism to infection of microorganism. It deals with the study processes by which the body defends itself from the invasion and attack of foreign organism.

The Immune System

- ✓ The immune system is the collection of cells, tissues and molecules that functions to defend us against infectious microbes. The coordinated reaction of the immune system against infections (and other foreign substances) is known as the immune response.
- ✓ Immunity is the state of protection against foreign organisms or substances (antigens).
- ✓ The immune system can be classified into two types such as the innate immune system and the adaptive immune system.
- ✓ An **immunogen** is a substance that induces a specific immune response.
- ✓ The immune system produces both humoral and cell-mediated responses.
- ✓ An **antigen** (Ag) is substance that reacts with the products of a specific immune response; i.e., antibodies or specific T cells.
- ✓ The **antigenic determinant** (or **epitope**) is that particular part of an antigen that combines the components of the specific immune response. This is important because it implies that (often ?) the truly antigenic part of an organism, might only be a very small part of the whole.
- ✓ A **hapten** is a substance, almost always very small in molecular terms, that by itself is non-immunogenic, but that can react with the products of a specific immune response. If a hapten is administered by itself, it cannot induce an immune response (thus, haptens are not immunogens). If the hapten combines with a carrier, then the combination is immunogenic. Also, free haptens can react with products of the immune response after such products have been formed (thus, haptens possess antigenicity).

- ✓ An **antibody** (Ab) is a specific protein (immunoglobulin) which is produced in response to an immunogen and which reacts with an antigen.
- ✓ Abnormalities of the immune system that result in defective immune responses make individuals susceptible to infections by viruses,bacteria, fungi and parasites.

The Lymphatic System

The Lymphatic System returns fluids that have leaked from the circulatory system back to the blood; protects the body by removing foreign material; provides a site for immune surveillance

Lymph

After blood travels through capillary beds and is moved to the venous system, some of its fluid is left behind in the tissues. This fluid and any plasma proteins (lymph) become part of the interstitial fluid and must be returned to the cardiovascular system. Lymphatic vessels are intimately connected with blood vessels so that they can perform this function.

Lymphoid cells

Lymphocytes

Lymphocytes are the central cells of the immune system, responsible for adaptive immunity and the immunologic attributes of diversity, specificity, memory, and self/non self recognition. Lymphocytes constitute 20%–40% of the body's white blood cells and 99% of the cells in the lymph. These lymphocytes continually circulate in the blood and lymph and are capable of migrating into the tissue spaces and lymphoid organs, thereby integrating the immune system to a high degree.

Lymphocytes are two types

- **T lymphocytes (T cells)**
  - Mature in the thymus

        - Directly attack and destroy foreign cells
    - **B lymphocytes (B cells)**
        - Mature in the bone marrow
        - Produce plasma cells that produce antibodies

Macrophages, Dendritic cells and Reticular cells is the antigen presenting cells, it initiate the immune response

Lymphocytes are one of the five kinds of **white blood cells** or **leukocytes** circulating in the blood.

Although mature lymphocytes all look pretty much alike, they are extraordinarily diverse in their functions. The most abundant lymphocytes are:

- **B lymphocytes** (often simply called **B cells**) and
- **T lymphocytes** (likewise called **T cells**).

B cells are produced in the **bone marrow**.

- The precursors of T cells are also produced in the bone marrow but leave the bone marrow and mature in the **thymus** (which accounts for their designation).
- Each B cell and T cell is **specific** for a particular **antigen**. What this means is that each is able to **bind to** a particular molecular structure.

The specificity of binding resides in a **receptor** for antigen:

- the B cell receptor (**BCR**) for antigen and
- the T cell receptor (**TCR**) respectively.

B cell surface marker

- **B220** (a form of CD45) is often used as a marker for B cells and their precursors.
- **Class II MHC** molecules allow the B cell to function as an antigen-presenting cell (APC).

- **CR1 (CD35) and CR2 (CD21**) are receptors for certain complement products.
- **Fc_RII (CD32**) is a receptor for IgG, a type of antibody.
- **B7-1 (CD80) and B7-2 (CD86**) are molecules that interact with CD28 and CTLA-4, important regulatory molecules on the surface of different types of T cells,including TH cells.
- **CD40** is a molecule that interacts with CD40 ligand on the surface of helper T cells.

T cell surface marker

CD28, a receptor for the co-stimulatory B7 family of molecules present on B cells and other antigenpresenting cells

CD45, a signal-transduction molecule

CD4-T cells generally function as T helper (TH) cells.

CD8_ T cells generally function as T cytotoxic (TC)cells.

Both BCRs and TCRs share these properties:

- They are integral membrane proteins.
- They are present in thousands of identical copies exposed at the cell surface.
- They are made before the cell ever encounters an antigen.
- They are encoded by genes assembled by the recombination of segments of DNA.
- They have a unique binding site.
- This site binds to a portion of the antigen called an antigenic determinant or **epitope**.
- The binding, like that between an **enzyme** and its **substrate** depends on complementarity of the surface of the receptor and the surface of the epitope.
- The binding occurs by non-covalent forces (again, like an enzyme binding to its substrate).
- Successful binding of the antigen receptor to the epitope, **if accompanied by additional signals**, results in:
    - stimulation of the cell to leave G_0 and enter the cell cycle.

- Repeated mitosis leads to the development of a **clone** of cells bearing the same antigen receptor; that is, a clone of cells of the identical specificity.

BCRs and TCRs differ in:

- their structure;
- the genes that encode them;
- the type of epitope to which they bind.

B Cells

- BCRs bind intact antigens (like diphtheria toxoid, the protein introduced into your body in the DTP vaccine). These may be
  - soluble molecules present in the extracellular fluid;
  - intact molecules that the B cell plucks from the surface of antigen-presenting cells like macrophages and dendritic cells.
- The bound antigen molecules are engulfed into the B cell by receptor-mediated endocytosis.
- The antigen is digested into fragments
- which are then displayed at the cell surface nestled inside a class II histocompatibility molecule.
- Helper T cells specific for this structure (i.e., with complementary TCRs) bind the B cell and
- secrete **lymphokines** that:
  - stimulate the B cell to enter the cell cycle and develop, by repeated mitosis, into a **clone** of cells with identical BCRs;
  - switch from synthesizing their BCRs as integral membrane proteins to a soluble version;
  - differentiate into **plasma cells** that secrete these soluble BCRs, which we now call **antibodies**.

T Cells

- The surface of each T cell also displays thousands of identical **T cell receptors** (**TCR**s).
- There are two types of T cells that differ in their TCR:
    - **alpha/beta** (αβ) T cells. Their TCR is a heterodimer of an alpha chain with a beta chain. Each chain has a variable (V) region and a constant (C) region. The V regions each contain 3 **hypervariable regions** that make up the antigen-binding site.
    - **gamma/delta** (γδ) T cells. Their TCR is also a heterodimer of a gamma chain paired with a delta chain.

The TCR (of alpha/beta T cells) binds a bimolecular complex displayed at the surface of **some other cell** called an **antigen-presenting cell** (**APC**). This complex consists of:

- a fragment of an antigen lying within the groove of a
- histocompatibility molecule

The complex has been compared to a "hot dog in a bun".

Most of the T cells in the body belong to one of two subsets. These are distinguished by the presence on their surface of **one or the other** of two glycoproteins designated:

- **CD4**
- **CD8**

Which of these molecules is present determines what types of cells the T cell can bind to.

- **$CD8^+$** T cells bind epitopes that are part of **class I histocompatibility molecules**. Almost all the cells of the body express class I molecules.
- **$CD4^+$** T cells bind epitopes that are part of **class II histocompatibility molecules**. Only specialized antigen-presenting cells express class II molecules. These include:
    - dendritic cells
    - phagocytic cells like **macrophages** and **B cells**

Antibodies:

A protein that is produced in response to an antigen

- Binds specifically to the antigen
- Form the class known as immunoglobulins
- Large family of soluble Glycoproteins
- Produced by B lymphocytes
- Found in serum
- Deficiency is life threatening
- After binding antigen, initiate secondary effector functions
  - Complement activation
  - Opsonisation
  - Cell activation via specific antibody-binding receptors (Fc receptors)

Function

In defence

- - targeting of infective organisms
- - recruitment of effector mechanisms
- - neutralisation of toxins
- - removal of antigens
- - passive immunity in the new born

In medicine

- - levels used in diagnosis and monitoring
- - pooled antibodies for passive therapy/protection

In laboratory science

- vast range of diagnostic and research applications

Lymphoid (lymphatic) tissues

- Function of lymphatic tissues
    - Provides a site of proliferation for lymphocytes
    - Surveillance site for lymphocytes and macrophages
- Composition of lymphatic tissues
    - Primarily composed of reticular connective tissue (except in the thymus)
    - Macrophages reside on the reticular fibers; lymphocytes temporarily reside in the spaces of the reticular fiber network before leaving to patrol the body
- **Types of lymphatic tissue organization**
    - Diffusse lymphatic tissue – scattered reticular tissue elements and cells, no capsule
        - Found in every body organ, especially in the basement membranes of mucous membranes and lymphoid organs
    - Lymphoid follicles (nodules) – solid spherical bodies of tightly packed reticular elements and cells
        - Typically have germinal centers
            - Contain dendritic cells and B cells

Lymphoid organs

The organs concerned with immune reaction are called lymphoid organ.The contain lymphoid cells (lymphocytes).The lymphoid organs and lymphoid cells constitute the lymphoid system.

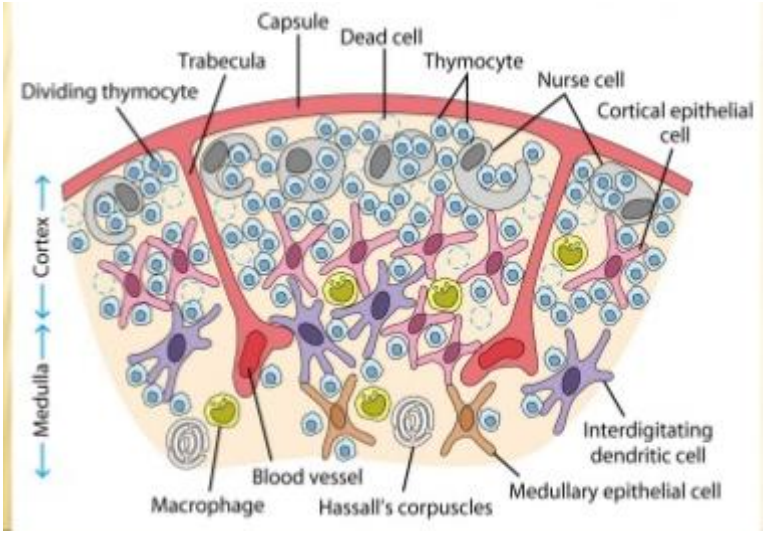
The primary or central lymphoid organs generate lymphocytes from immature progenitor cells. The thymus and the bone marrow constitute the primary lymphoid organs involved in the production and early clonal selection of lymphocyte tissues. Bone marrow is responsible for both the creation of T cells and the production and maturation of B cells.

Secondary or peripheral lymphoid organs, which include lymph nodes and the spleen, maintain mature naive lymphocytes and initiate an adaptive immune response. The peripheral lymphoid organs are the sites of lymphocyte activation by antigens. Activation leads to clonal expansion and affinity maturation. Mature lymphocytes recirculate between the blood and the peripheral lymphoid organs until they encounter their specific antigen.

The primary or central lymphoid organs

Thymus

- It is the site of T cell maturation
- Most active in younger children; atrophies with age
- Does not contain reticular fibers
- Lack B cells, therefore no germinal centers are present in the thymus



It is a flat, bilobed organ situated above the heart. Each lobe is surrounded by a capsule and is divided into **lobules**, which are separated from each other by strands of connective tissue called **trabeculae**. Each lobule is organized into two compartments: the outer compartment, or **cortex**, is densely packed with immature T cells, called thymocytes, whereas the inner compartment, or **medulla,** is sparsely populated with thymocytes.

Both the cortex and medulla of the thymus are crisscrossedby a three-dimensional stromal-cell network composed of epithelial cells, dendritic cells, and macrophages. The medulla consists of MHC class II antigen. In addition there are some peculiar strcture in the medulla called **Hassall's corpucles.** The function of the thymus is to generate and select a repertoire of T cells that will protect the body from infection.

Function

- The function of the thymus is to generate and select a repertoire of T cells that will protect the body from infection.
- It bring about cell mediated immunity.
- It bring about graft rejection.

Bone marrow

Bone marrow is the spongy tissue inside your **bones**. It's home to blood vessels and stem cells that help produce red and white blood cells and Platelets. **Bone marrow** is responsible for both the creation of T cells and the production and maturation of B cells.

There are two types of bone marrow:

1.Vascular and adipose region

2.Haemopoitic region

The **Vascular region** is the circulatory system that supplies nutrient and removes waste from the actively growing blood cells .The other half of the bone marrow which is actively involved in the haemopoisis is known as the red marrow.

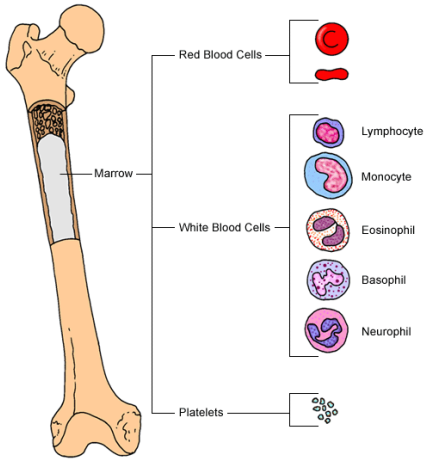
R**ed marrow** (consisting mainly of with proliferating and differentiating blood cells in connective tissue matrices bordered by venous sinuses, Red blood cells, platelets and most white blood cells (Plasma cells and macrophages) arise in red marrow) .In adult animals much of the red marrow is replaced by fatty tissue and become **Yellow marrow**.

Main function of bone Marrow.

1. It is the site of origin of all T and B cells, Phagocytes, platelets, erythrocytes and other leukocytes in adults. All the cells of the blood, including lymphocytes, are produced from hemopoiteic stem cells (HSC) to give rise all elements of the blood.

2. In addition to hematopoiesis. Bone marrow is the site of removal of aged and defective erythrocyte.

3. Site of differentiation and maturation of B-lymphocytes. In bone marrow, the B-cells develop their receptors during different stages in bone marrow



Secondary or peripheral lymphoid organs

Lymph nodes

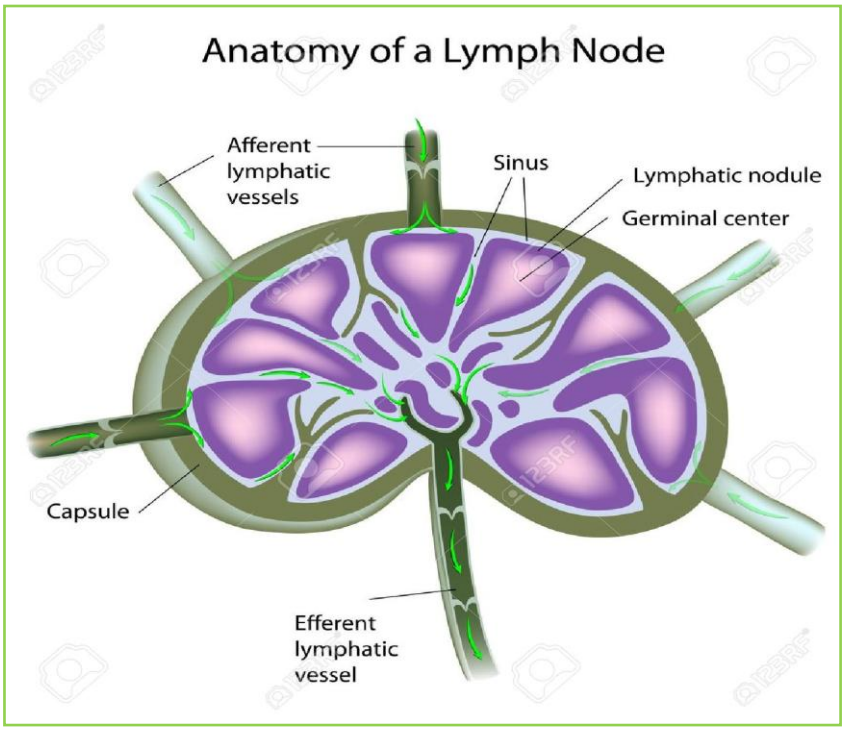
- Only the lymph nodes filter lymph
- Cluster along the lymphatic vessels of the body
- Lymph is filtered through the lymph nodes before it is returned to the bloodstream
- Lymph nodes are embedded in connective tissue
- Large clusters of lymph nodes appear near the body surface in the inguinal, axillary, and cervical regions.
- Functions of lymph nodes
- Filters lymph
- Assist in activating the immune system

Anatomy of a lymph node

Lymph nodes are the sites where immune responses are mounted to antigens in lymph. They are encapsulated beanshaped structures containing a reticular network packed with lymphocytes, macrophages, and dendritic cells.

Lymph nodes is coveredby a capsule.The capsule penetrate into the lymph node to form septa called trabeculae(Strands of connective tissue which divide the node into compartments). Morphologically, a lymph node can be divided into three roughly concentric regions: the cortex, the paracortex, and the medulla, each of which supports a distinct microenvironment. The outermost layer, the cortex, contains lymphocytes (mostly B cells), macro-phages, and follicular dendritic cells arranged in primary follicles. After antigenic challenge, the primary follicles enlarge into secondary follicles, each containing a germinal center.

Beneath the cortex is the paracortex, which is populated largely by T lymphocytes and also contains interdigitating dendritic cells thought to have migrated from tissues to the node. These interdigitating dendritic cells express high levels of class II MHC molecules,which are necessary for presenting antigen to TH cells.



The innermost layer of a lymph node, the medulla, is more sparsely populated with lymphoid-lineage cells; of those present, many are plasma cells actively secreting antibody molecules.

- Circulation in the lymph nodes
- Afferent lymphatic vessels – lymph enters here
- Once inside the nodes, the lymph moves through a series of sinuses and then exits at the hilus
- Efferent lymphatic vessels – lymph exits here

As antigen is carried into a regional node by the lymph, itis trapped, processed, and presented together with class II MHC molecules by interdigitating dendritic cells in the paracortex, resulting in the activation of TH cells. The initial activation of B cells is also thought to take place within the T-cell-rich paracortex. Once activated, TH and B cells form small foci consisting largely of proliferating B cells at the edges of the paracortex.

Some B cells within the foci differentiate into plasma cells secreting IgM and IgG. These foci reach maximum size within 4–6 days of antigen challenge. Within 4–7 days of antigen challenge, a few B cells and TH cells migrate to the primary follicles of the cortex. It is not known what causes this migration.Within a primary follicle, cellular interactions between follicular dendritic cells, B cells, and TH cells take place, leading to development of a secondary follicle with a central germinal center. Some of the plasma cells generated in the germinal center move to the medullary areas of the lymph node, and many migrate to bone marrow.

Afferent lymphatic vessels pierce the capsule of a lymph node at numerous sites and empty lymph into the subcapsular sinus. Lymph coming from the tissues percolates slowly inward through the cortex, paracortex, and medulla, allowing phagocytic cells and dendritic cells to trap any bacteria or particulate material (e.g., antigen-antibody complexes) carried by the lymph.

After infection or the introduction of other antigens into the body, the lymph leaving a node through its single efferent lymphatic vessel is enriched with antibodies newly secreted by medullary plasma cells and also has a fiftyfold higher concentration of lymphocytes than the afferent lymph.

Spleen

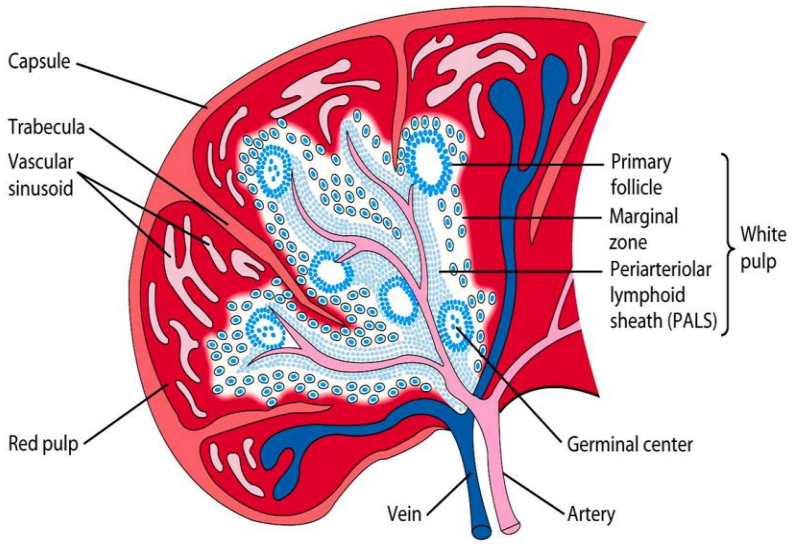
The spleen plays a major role in mounting immune responses to antigens in the blood stream. It is a large, ovoid secondary lymphoid organ situated high in the left abdominal cavity. While lymph nodes are specialized for trapping antigen from local tissues, the spleen specializes in filtering blood and trapping blood-borne antigens; thus, it can respond to systemic infections.

Functions of the spleen

- Cleanses the blood by removing old RBCs and platelets, as well as debris from the blood.
- Stores the breakdown products of RBCs
- Site of erythrocyte production in the fetus

Anatomy of the spleen

- Surrounded by a fibrous capsule
- Contains both T cells, B cells, RBCs and macrophages



- Divided histologically into two regions

Red pulp

- It consists of a network of sinusoids populated by macrophages and numerous red blood cells (erythrocytes) and few lymphocytes;
- it is the site where old and defective red blood cells are destroyed and removed

White pulp

The splenic white pulp surrounds the branches of the splenic artery, forming a periarteriolar lymphoid sheath (PALS) populated mainly by T lymphocytes. Primary lymphoid follicles are attached to the PALS. These follicles are rich in B cells and some of them contain germinal centers. The marginal zone, located peripheral to the PALS, is populated by lymphocytes and macrophages.

Function

Blood-borne antigens and lymphocytes enter the spleen through the splenic artery, which empties into the marginal zone. In the marginal zone, antigen is trapped by interdigitating dendritic cells, which carry it to the PALS.

Lymphocytes in the blood also enter sinuses in the marginal zone and migrate to the PALS. The initial activation of B and T cells takes place in the Tcell- rich PALS. Here interdigitating dendritic cells capture antigen and present it combined with class II MHC molecules to TH cells. Once activated, these TH cells can then activate B cells.

The activated B cells, together with some TH cells, then migrate to primary follicles in the marginal zone. Upon antigenic challenge, these primary follicles develop into characteristic secondary follicles containing germinal centers (like those in the lymph nodes), where rapidly dividing B cells (centroblasts) and plasma cells are surrounded by dense clusters of concentrically arranged lymphocytes.

Tonsils – the simplest lymphoid organs; named according to their location

- Palatine tonsils

        - Lingual tonsils
        - Pharyngeal tonsil
        - Tubal tonsils
- Aggregates of lymphoid follicles
    - Location of these follicles make them ideal because they are able to:
        - Detroy bacteria and prevent pathogens from slipping through the intestinal wall
        - Generate many "memory" lymphocytes for long-term immunity
    - Examples
        - **Peyer's patches** – found in the distal portion of the small intestine
        - **Appendix** – an off-shoot of the cecum (the first part of the large intestine)
- **Mucosa-associated lymphatic tissue (MALT)** – protects the digestive and respiratory tracts from foreign material
    - Include the tonsils, Peyer's patches, appendix, and lymphoid follicles found in the bronchi

Clinical Disorders of the Lymphatic System

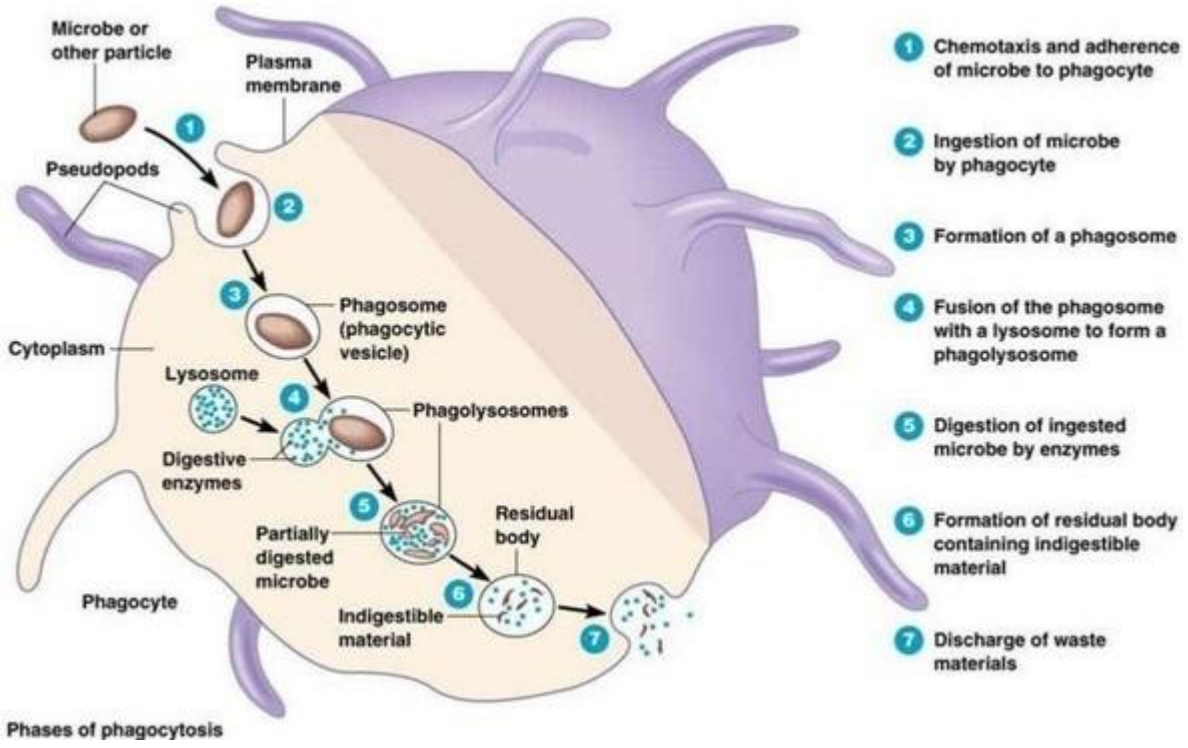
- Lymphangitis – inflammation of the lymphatic vessels
- Lymphedema – blockage of the lymphatic vessels
- Buboes - infected lymph nodes

Phagocytosis

- Microbes that penetrate the first line of defense face the second line of defense, which depends mainly on **phagocytosis,** the ingestion of invading organisms by certain types of white cells. The main phagocytic cells are polymorphonuclear neutrophils and macrophages.
- Phagocyte function is intimately associated with an effective inflammatory response and also with certain antimicrobial proteins.
- Phagocytes attach to their prey via surface receptors found on microbes but not normal body cells.

- Adherence induces membrane protrusions, called pseudopodia, Fusion of the pseudopodia encloses the material within a membrane-bounded structure called a phagosome, a phagosome moves toward the cell interior,where it fuses with a lysosome to form a phagolysosome.

Stages of phagocytosis



- Microbes are destroyed within lysosomes in two ways.
  - Lysosomes contain nitric oxide and other toxic forms of oxygen, which act as potent antimicrobial agents.
  - Lysozymes and other enzymes degrade mitochondrial components.

- Some microbes have adaptations that allow them to evade destruction by phagocytes.

- The outer capsule of some bacterial cells hides their surface polysaccharides and prevents phagocytes from attaching to them.
- Other bacteria are engulfed by phagocytes but resist digestion, growing and reproducing within the cells.

- Four types of white blood cells are phagocytic.
- The phagocytic cells called **neutrophils** constitute about 60–70% of all white blood cells (leukocytes).
  - Cells damaged by invading microbes release chemical signals that attract neutrophils from the blood.
  - The neutrophils enter the infected tissue, engulfing and destroying microbes there.
  - Neutrophils tend to self-destruct as they destroy foreign invaders, and their average life span is only a few days.
- **Monocytes,** about 5% of leukocytes, provide an even more effective phagocytic defense.
  - After a few hours in the blood, they migrate into tissues and develop into **macrophages,** which are large, long-lived phagocytes.
  - Some macrophages migrate throughout the body, while others reside permanently in certain tissues, including the lungs, liver, kidneys, connective tissues, brain, and especially in lymph nodes and the spleen.
- The fixed macrophages in the spleen, lymph nodes, and other **lymphatic** tissues are particularly well located to contact infectious agents.
  - Microbes that enter the blood become trapped in the spleen, while microbes in interstitial fluid flow into lymph and are trapped in lymph nodes.
  - In either location, microbes soon encounter resident macrophages.
- **Eosinophils,** about 1.5% of all leukocytes, contribute to defense against large parasitic invaders, such as the blood fluke, Schistosoma mansoni.
  - Eosinophils position themselves against the external wall of a parasite and discharge destructive enzymes from cytoplasmic granules.

- **Dendritic cells** can ingest microbes like macrophages. However, their primary role is to stimulate the development of acquired immunity.
- A variety of proteins function in innate defense either by attacking microbes directly or by impeding their reproduction.
    - In addition to lysozyme, other antimicrobial agents include about 30 serum proteins, known collectively as the **complement system.**
        - Substances on the surface of many microbes can trigger a cascade of steps that activate the complement system, leading to lysis of microbes.
- Another set of proteins that provide innate defenses are the **interferons,** which defend against viral infection.
    - These proteins are secreted by virus-infected body cells and induce uninfected neighboring cells to produce substances that inhibit viral reproduction.
    - Interferon limits cell-to-cell spread of viruses, helping to control viral infection.
    - Because they are nonspecific, interferons produced in response to one virus may confer short-term resistance to unrelated viruses.
    - Interferons make increases in the expression of class I and class II MHC molecules, and boost the NK-cell activity.
    - These are three types

      IFN α- is an effective treatment for this leukemia

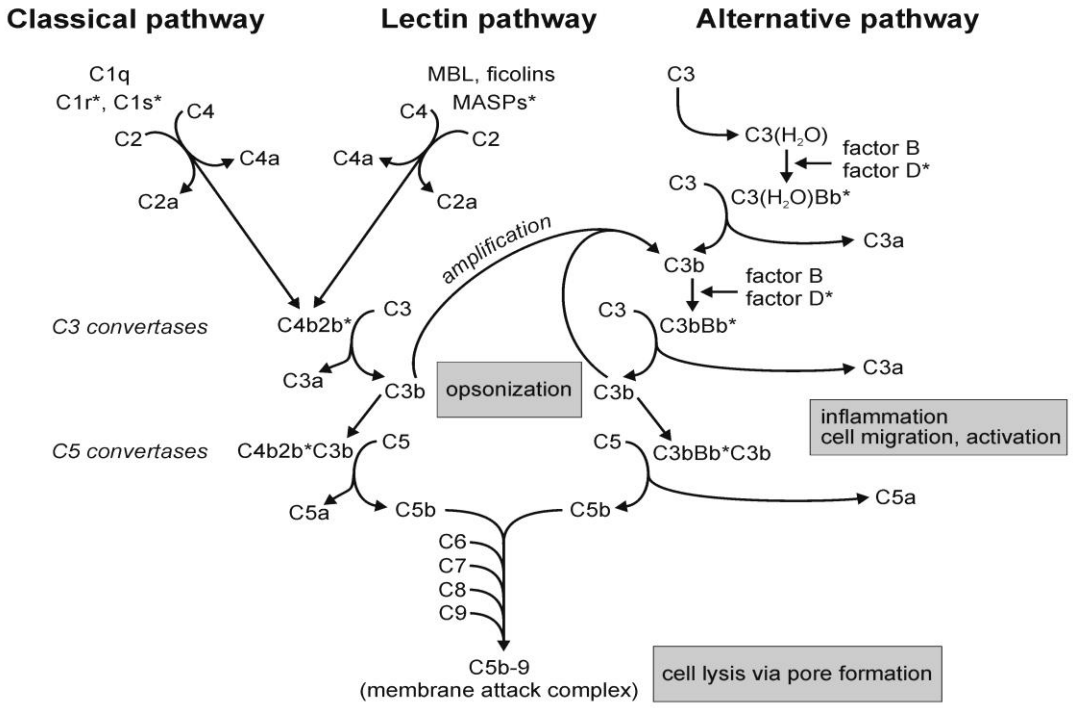
      IFN β-is an treatment for Multiple sclerosis

      IFN γ- is used in the treatment of inflammatory response.

Complement:

- The complement system is the major effector of the humoral branch of the immune system.
- The proteins and glycoproteins that compose the complement system are synthesized mainly by liver hepatocytes, and blood monocytes, tissue macrophages ,and epithelial cells of the gastrointestinal and genitourinary tracts.

- The complement system comprises a group of serum proteins,many of which exist in inactive forms.
- Complement activation occurs by the classical, alternative,or lectin pathways, each of which is initiated differently.The three pathways converge in a common sequence of events that leads to generation of a molecular complex that causes cell lysis.
- Activation of the alternative and lectin pathways is antibody-independent. These pathways are initiated by reaction of complement proteins with surface molecules of microorganisms.
- The classical pathway is initiated by antibody binding to a cell target; reactions of IgM and certain IgG subclasses activate this pathway.
- complement deficiencies range from increases in susceptibility to infection to tissue damage caused by immune complexes.



Function of complement

- ❖ Lysis of cells, bacteria, and viruses
- ❖ Opsonization, which promotes phagocytosis of particulate antigens
- ❖ Binding to specific complement receptors on cells of the immune system, triggering specific cell functions,
- ❖ Inflammation, and secretion of immunoregulato molecules

- ❖ Immune clearance, which removes immune complexe from the circulation and deposits them in the spleen and liver

Immune Tolerance (Ray D. Owens in 1945)

- **Definition:** an active physiological process producing **immunological unresponsiveness** to an otherwise immunogenic substance
- both humoral and CMI must be inhibited
- depends upon both **dose** and **presentation**,
  - high dose produces tolerance in T-cells and B-cells
  - low dose produces tolerance in T-cells only
  - monomeric solutions may produce tolerance where macromolecules are immunogenic
- requires repeat exposure
- is easier to produce in the neonate than in the adult
- mechanism involves the presence of T-suppressor cells which are **antigen specific**, or the presence of antibodies which alter self-antigens such that they are no longer susceptible to an immune response.

Unit II-Immunity and Immune response

Overview: Reconnaissance, Recognition, and Response

- An animal must defend itself against unwelcome intruders—the many potentially dangerous viruses, bacteria, and other pathogens it encounters in the air, in food, and in water.
- It must also deal with abnormal body cells, which, in some cases, may develop into cancer.
- Two major kinds of defense have evolved to counter these threats.
- The first kind of defense is **innate immunity**.
    - Innate defenses are largely **nonspecific**, responding to a broad range of microbes.
    - Innate immunity consists of external barriers formed by the skin and mucous membranes, plus a set of internal cellular and chemical defenses that defend against microbes that breach the external barriers.
    - The internal defenses include macrophages and other phagocytic cells that ingest and destroy pathogens.
- A second kind of defense is **acquired immunity.**
    - Acquired immunity develops only after exposure to microbes, abnormal body cells, or other foreign substances.
    - Acquired defenses are highly **specific** and can distinguish one inducing agent from another.
    - This recognition is achieved by white blood cells called **lymphocytes,** which produce two general types of immune responses.

Innate immunity provides broad defenses against infection

- An invading microbe must penetrate the external barrier formed by the skin and mucous membranes, which cover the surface and line the openings of an animal's body.
- If it succeeds, the pathogen encounters the second line of nonspecific defense, innate cellular and chemical mechanisms that defend against the attacking foreign cell.

The skin and mucous membrane provide first-line barriers to infection.

- Intact skin is a barrier that cannot normally be penetrated by bacteria or viruses, although even minute abrasions may allow their passage.
- Likewise, the mucous membranes that line the digestive, respiratory, and genitourinary tracts bar the entry of potentially harmful microbes.
    - Cells of these mucous membranes produce mucus, a viscous fluid that traps microbes and other particles.
    - In the trachea, ciliated epithelial cells sweep out mucus with its trapped microbes, preventing them from entering the lungs.
- Beyond their role as a physical barrier, the skin and mucous membranes counter pathogens with chemical defenses.
    - In humans, for example, secretions from sebaceous and sweat glands give the skin a pH ranging from 3 to 5, which is acidic enough to prevent colonization by many microbes.
    - Microbial colonization is also inhibited by the washing action of saliva, tears, and mucous secretions that continually bathe the exposed epithelium.
        - All these secretions contain antimicrobial proteins.
        - One of these, the enzyme **lysozyme,** digests the cell walls of many bacteria, destroying them.
- Microbes present in food or water, or those in swallowed mucus, must contend with the highly acidic environment of the stomach.
    - The acid destroys many microbes before they can enter the intestinal tract.
    - One exception, the virus hepatitis A, can survive gastric acidity and gain access to the body via the digestive tract.

Primary Response

- thymus dependent
    - IgM is first Ab to appear, with a peak ~ 2 weeks
    - switch from IgM ---> IgG / IgA / IgE requires T-cell co-operation
- thymus independent

- IgM is the **only** Ab to appear

Secondary Response

- occurs earlier than the primary response, usually within 4-5 days
- marked proliferation of Ab producing and effector T-cells
- Ab is usually IgG and has a higher affinity, and therefore more specific
- requires immunological memory in both T-cells and B-cells

In acquired immunity, lymphocytes provide specific defenses against infection

- While microorganisms are under assault by phagocytic cells, the inflammatory response, and antimicrobial proteins, they inevitably encounter lymphocytes, the key cells of acquired immunity, the body's second major kind of defense.
- As macrophages and dendritic cells phagocytose microbes, they secrete certain cytokines that help activate lymphocytes and other cells of the immune system.
  - Thus the innate and acquired defenses interact and cooperate with each other.
- Any foreign molecule that is recognized by and elicits a response from lymphocytes is called an **antigen.**
  - Most antigens are large molecules such as proteins or polysaccharides.
  - Most are cell-associated molecules that protrude from the surface of pathogens or transplanted cells.
  - A lymphocyte actually recognizes and binds to a small portion of an antigen called an **epitope.**

The Immune response

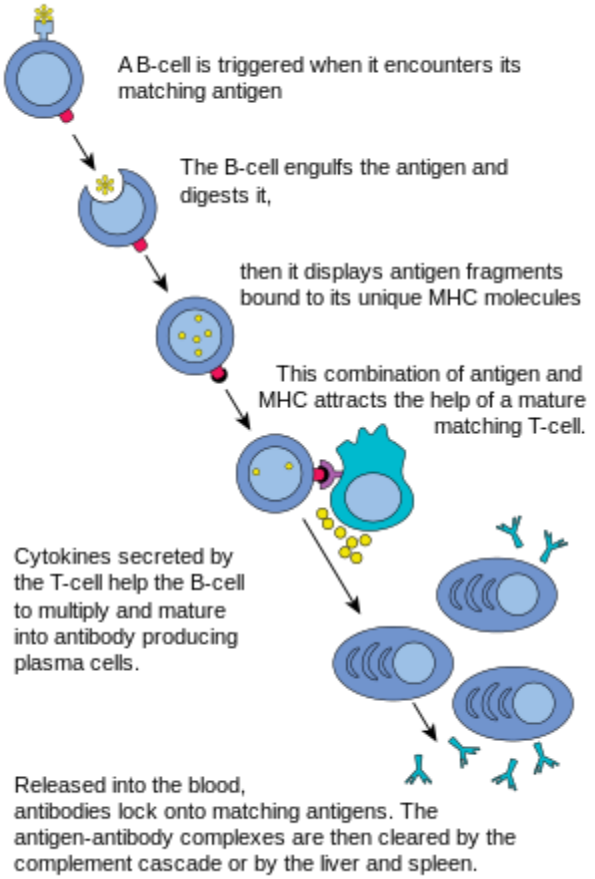
- Introduction of a foreign substance may produce,
  - specific antibody(Humoral immune response)
  - cell mediated immunity

Humoral immune response:

- **The Humoral immunity** refers to antibody mediated immune response .In the humoral response, cells derived from B-lymphocytes secrete defensive proteins called antibodies that bind to microbes and target them for elimination. The Humoral response is suited for elimination of exogenous antigens. Humoral immunity is named because it involves substances found in the humours, or body fluids.

The humoral immune response is mediated by B cell.

Steps involved in Humoral immune response:



- **B cell activation**-Antigen binding to Native B cell receptor
- **Clonal Selection**-Activated Bcell undergo cell division.The phenomenon of selective proliferation of Bcell in response to their interaction with the antigen is called Clonal Selection.

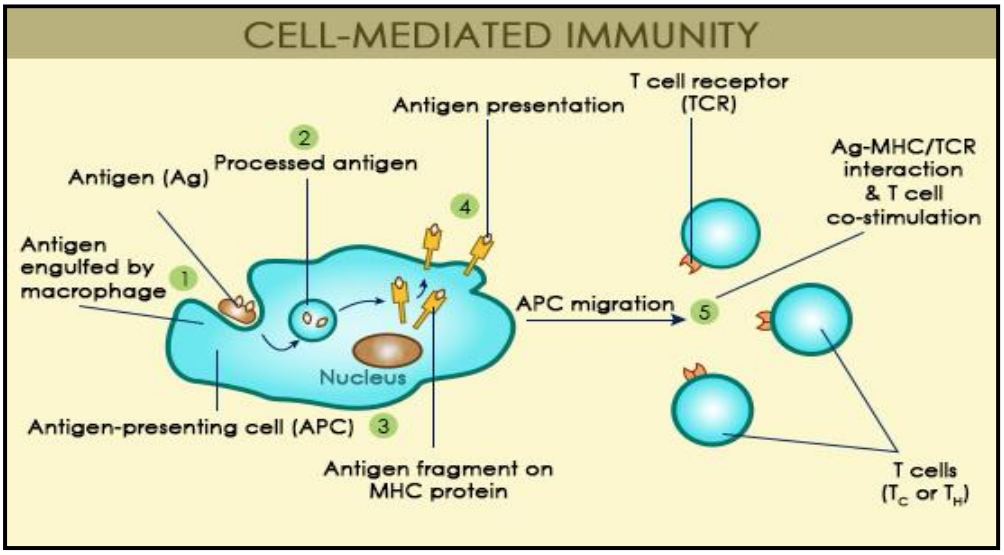
- **Differentiation**-Activated B cell produce Plasma cell and Memory cell.
- **Ab production**-Plasma cell produce Antibodies that bind to antigen and Memory cell for immunological memory or secondary response.

Cell mediated immunity:

- Cell-mediated immunity is an immune response that does not involve antibodies, but rather involves the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen.
- Tcells interact with other immune cells is via a receptor is called T cell receptor(TCR),which can only interact with antigenic molecule bound to class to molecule on the surface of antigenic presenting cell(APC_S).
- The cell-mediated immunity involves the activation of NK cells, T lymphocytes, macrophages, and cytokines in response to an antigen.

Cellular immunity protects the body by:

- activating antigen-specific cytotoxic T-lymphocytes that are able to induce apoptosis in body cells displaying epitopes of foreign antigen on their surface, such as virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumor antigens;
- activating macrophages and natural killer cells, enabling them to destroy pathogens; and
- stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive immune responses and innate immune responses.



Steps:

1. The process starts when an antigen presenting cell, usually a macrophage, ingests foreign material and incorporates a piece of the antigen into the surface of the cell membrane.

2. Everywhere the macrophage then travels, it can present this antigen to any T-Helper cells that it encounters.

3. The T-Helper cells have a receptor in their membrane cleverly called the T-Cell Receptor. This is a protein receptor with a specific shape. If the antigen on the macrophage matches with a T-cell receptor on the T-Helper cell, it becomes an active T-cell. There are literally millions of different T-helper cells with different receptor shapes. Only when the macrophage finds the specific, appropriate one will the process continue. That's why it takes some time before the immune system starts to respond the infection. Image you as a single person being tasked with speaking with everyone on campus until you spoke with JoePa. It's possible but it would take some time.

4. Once the appropriate T-helper cell is activated, it can now proceed to stimulate another T-cell.

5. The Cytotoxic T-cell, once stimulated by a t-helper cell, will search out and destroy any cells in the body that are misbehaving. Any cell with an altered MHC, self identity protein will be targeted. If a cell has the wrong MHC, like a transplanted kidney cell it can be attacked. When cells are invaded by viruses or become cancerous, the MHC markers become altered as the cell is fixated on other inappropriate tasks. This altered marker can also be recognized by Cytotoxic T-cells and destroyed. In this way the problem is eliminated.

6. Once the active infection or tumor or transplant is destroyed many of the T cells, both helper and cytotoxic, will slowly die off. The problem is over, they're no longer needed. However, a number of them remain as Memory cells. These cells will respond to the same antigen if it is ever encountered again. In this way, following viral infections, for example, are easier to fight as there are multiple T- helper cells ready to respond. The macrophage does not have to search the whole body for a single T-helper cell, there are now many. Again using the earlier analogy,

instead of you yourself trying to find JoePa, imagine you and all your friends splitting up and looking in different buildings on campus. The job would be easier and faster.

Lymphocytes provide the specificity and diversity of the immune system.

- The vertebrate body is populated by two main types of lymphocytes: **B lymphocytes (B cells)** and **T lymphocytes (T cells).**
  - Both types of lymphocytes circulate throughout the blood and lymph and are concentrated in the spleen, lymph nodes, and other lymphatic tissue.
- B and T cells recognize antigens by means of antigen-specific receptors embedded in their plasma membranes.
  - A single B or T cell bears about 100,000 identical **antigen receptors.**
- Because lymphocytes recognize and respond to particular microbes and foreign molecules, they are said to exhibit specificity for a particular epitope on an antigen.
- Each **B cell receptor** for an antigen is a Y-shaped molecule consisting of four polypeptide chains: two identical **heavy chains** and two identical **light chains** linked by disulfide bridges.
  - A region in the tail portion of the molecule, the transmembrane region, anchors the receptor in the cell's plasma membrane.
  - A short region at the end of the tail extends into the cytoplasm.
- At the two tips of the Y-shaped molecules are the light- and heavy-chain variable (V) regions whose amino acid sequences vary from one B cell to another.
- The remainder of the molecule is made up of the constant (C) regions, which do not vary from cell to cell.
- Each B cell receptor has two identical antigen-binding sites formed from part of a heavy-chain V region and part of a light-chain V region.
- The interaction between an antigen-binding site and its corresponding antigen is stabilized by multiple noncovalent bonds.

- Secreted antibodies, or **immunoglobulins,** are structurally similar to B cell receptors but lack the transmembrane regions that anchor receptors in the cell membrane.
    - B cell receptors are often called membrane antibodies or membrane immunoglobulins.
- Each **T cell receptor** for an antigen consists of two different polypeptide chains: an alpha chain and a beta chain, linked by a disulfide bridge.
- Near the base of the molecule is a transmembrane region that anchors the molecule in the cell's plasma membrane.
- At the outer tip of the molecule, the alpha and beta chain variable (V) regions form a single antigen-binding site.
- The remainder of the molecule is made up of the constant (C) regions.
- T cell receptors recognize and bind with antigens with the same specificity as B cell receptors.
- However, while the receptors on B cells recognize intact antigens, the receptors on T cells recognize small fragments of antigens that are bound to normal cell-surface proteins called MHC molecules.
- MHC molecules are encoded by a family of genes called the **major histocompatibility complex (MHC).**
- As a newly synthesized MHC molecule is transported toward the plasma membrane, it binds with a fragment of antigen within the cell and brings it to the cell surface, a process called **antigen presentation.**
- There are two ways in which foreign antigens can end up inside cells of the body.
    - Depending on their source, peptide antigens are handled by a different class of MHC molecule and recognized by a particular subgroup of T cells.
        - **Class I MHC molecules,** found on almost all nucleated cells of the body, bind peptides derived from foreign antigens that have been synthesized within the cell.
            - Any body cell that becomes infected or cancerous can display such peptide antigens by virtue of its class I MHC molecules.

        - Class I MHC molecules displaying bound peptide antigens are recognized by a subgroup of T cells called **cytotoxic T cells.**
    - **Class II MHC molecules** are made by dendritic cells, macrophages, and B cells.
        - In these cells, class II MHC molecules bind peptides derived from foreign materials that have been internalized and fragmented by phagocytosis.

- For each vertebrate species, there are numerous different alleles for each class I and class II MHC gene, producing the most polymorphic proteins known.
    - As a result of the large number of different alleles in the human population, most of us are heterozygous for every one of our MHC genes.
    - Moreover, it is unlikely that any two people, except identical twins, will have exactly the same set of MHC molecules.
    - The MHC provides a biochemical fingerprint virtually unique to each individual that marks body cells as "self."

Lymphocyte development gives rise to an immune system that distinguishes self from nonself.

- Lymphocytes, like all blood cells, originate from pluripotent stem cells in the bone marrow or liver of a developing fetus.
- Early lymphocytes are all alike, but they later develop into T cells or B cells, depending on where they continue their maturation.
- Lymphocytes that migrate from the bone marrow to the **thymus** develop into T cells.
- Lymphocytes that remain in the **bone marrow** and continue their maturation there become B cells.
- There are three key events in the life of a lymphocyte.
    - The first two events take place as a lymphocyte matures, before it has contact with any antigen.

- The third event occurs when a mature lymphocyte encounters and binds a specific antigen, leading to its activation, proliferation, and differentiation—a process called **clonal selection.**

- The variable regions at the tip of each antigen receptor chain, which form the antigen-binding site, account for the diversity of lymphocytes.
  - The variability of these regions is enormous.
  - Each person has as many as a million different B cells and 10 million different T cells, each with a specific antigen-binding ability.

- At the core of lymphocyte diversity are the unique genes that encode the antigen receptor chains.
  - These genes consist of numerous coding gene segments that undergo random, permanent rearrangement, forming functional genes that can be expressed as receptor chains.
  - Genes for the light chain of the B cell receptor and for the alpha and beta chains of the T cell receptor undergo similar rearrangements, but we will consider only the gene coding for the light chain of the B cell receptor.
  - The immunoglobulin light-chain gene contains a series of 40 variable (V) gene segments separated by a long stretch of DNA from 5 joining (J) gene segments.
  - Beyond the J gene segments is an intron, followed by a single exon that codes for the constant region of the light chain.
  - In this state, the light-chain gene is not functional.
  - However, early in B cell development, a set of enzymes called recombinase link one V gene segment to one J gene segment, forming a single exon that is part V and part J.
    - Recombinase acts randomly and can link any one of 40 V gene segments to any one of 5 J gene segments.
    - For the light-chain gene, there are 200 possible gene products (20 V × 5 J).
    - Once V-J rearrangement has occurred, the gene is transcribed and translated into a light chain with a variable and constant region. The light chains combine randomly with the heavy chains that are similarly produced.

- The random rearrangements of antigen receptor genes may produce antigen receptors that are specific for the body's own molecules.
- As B and T cells mature, their antigen receptors are tested for potential self-reactivity.
- Lymphocytes bearing receptors specific for molecules present in the body are either destroyed by apoptosis or rendered nonfunctional.
  - Failure to do this can lead to autoimmune diseases such as multiple sclerosis.

Antigens interact with specific lymphocytes, inducing immune responses and immunological memory.

- Although it encounters a large repertoire of B cells and T cells, a microorganism interacts only with lymphocytes bearing receptors specific for its various antigenic molecules.
- A lymphocyte is "selected" when it encounters a microbe with epitopes matching its receptors.
  - Selection activates the lymphocyte, stimulating it to divide and differentiate, and eventually to produce two clones of cells.
  - One clone consists of a large number of **effector cells,** short-lived cells that combat the same antigen.
  - The other clone consists of **memory cells,** long-lived cells bearing receptors for the same antigen.
- This antigen-driven cloning of lymphocytes is called **clonal selection** and is fundamental to acquired immunity.
  - Each antigen, by binding selectively to specific receptors, activates a tiny fraction of cells from the body's diverse pool of lymphocytes.
  - This relatively small number of selected cells gives rise to clones of thousands of cells, all specific for and dedicated to eliminating that antigen.
- The selective proliferation and differentiation of lymphocytes that occur the first time the body is exposed to an antigen is the **primary immune response.**
  - About 10 to 17 days are required from the initial exposure for the maximum effector cell response.

- During this period, selected B cells and T cells generate antibody-producing effector B cells called **plasma cells,** and effector T cells, respectively.
- While this response is developing, a stricken individual may become ill, but symptoms of the illness diminish and disappear as antibodies and effector T cells clear the antigen from the body.

- A second exposure to the same antigen at some later time elicits the **secondary immune response.**
  - This response is faster (only 2 to 7 days), of greater magnitude, and more prolonged.
  - In addition, the antibodies produced in the secondary response tend to have greater affinity for the antigen than those secreted in the primary response.
- Measures of antibody concentrations in the blood serum over time show the difference between primary and secondary immune responses.
  - The immune system's capacity to generate secondary immune responses is called immunological memory, based not only on effector cells, but also on clones of long-lived T and B memory cells.
    - These memory cells proliferate and differentiate rapidly when they later contact the same antigen.

Unit III- Hypersensitivity and autoimmunity

Hypersensitivity

- Hypersensitive or allergy reactions are inflammatory reactions within the humoral or cell-mediated branches of the immune system that lead to wide tissue damage or even death.
- It mediated by Several compound is called as Mediator.
- The mediators can be classified as either primary or secondary.
- Primary mediators are histamine, proteases,eosinophil chemotactic factor, neutrophil chemotactic factor and heparin.
- Secondary mediators include platelet-activating factor, leukotrienes, prostaglandins, bradykinins, and various cytokines.

It classified in to four types

- Type I hypersensitive reaction is mediated by IgE antibodies.
- Type II hypersensitive reaction occurs when antibody(IgD) reacts with antigenic determinants present on the surface of cells, leading to cell damage or death through complement mediated lysis or antibody-dependent cell-mediated cytotoxicity(ADCC).
- Type III hypersensitive reaction is mediated by the formation of immune complexes and the ensuing activation of complement.
- Type IV hypersensitive reaction involves the cell-mediated branch of the immune system.

Types of Hyper Sensitivity

Type of hypersensitivity	Pathologic Immune Mechanisms	Mechanisms of tissue injury and disease
Immediate hypersensitivity: Type I	IgE antibody	Mast cells and their mediators (vasoactive amines, lipid mediators, cytokines)
Antibody mediated: Type II	IgM, IgG antibodies against cell surface or extracellular matrix antigens	Opsonization and phagocytosis of cells Complement- and Fc receptor-mediated recruitment and activation of leukocytes (neutrophils, macrophages) Abnormalities in cellular functions, e.g., hormone receptor signaling
Immune complex mediated: Type III	Immune complexes of circulating antigens and IgM or IgG antibodies	Complement- and Fc receptor–mediated recruitment and activation of leukocytes
T cell mediated: Type IV	1. $CD4^+$ T cells (delayed-type hypersensitivity) 2. $CD8^+$ CTLs (T cell–mediated cytolysis)	1. Macrophage activation, cytokine-mediated inflammation 2. Direct target cell killing, cytokine-mediated inflammation

Abbreviations: CTL, cytolytic T lymphocyte

Autoimmune Disease

- They result in an improper response of the immune system against self-components termed **autoimmunity**.
- Autoimmune diseases can be divided into organspecific and systemic diseases.
- The organ-specific diseases involve an autoimmune response directed mainly against a single organ or gland.
- The systemic diseases are directed against a broad spectrum of tissues and have manifestations in a variety of organs resulting from cell-mediated responses and cellular damage caused by auto-antibodies or immune complexes.

Autoimmune disease may be due to,

- failure of suppression - ie. a T-cell defect
- tissue damage altering self-antigens with a sustained response
- classical example is post-streptococcal GN
- infection altering cell surface markers in a genetically susceptible individual
- IDDM probably included in this group

Organ-Specific Autoimmune Diseases

- Addison's disease-Adrenal cells
- Goodpasture's syndrome-Renal and lung basement membranes
- Graves' disease-Thyroid-stimulating hormone receptor
- Hashimoto's thyroiditis-Thyroid proteins and cell
- Myasthenia gravis-Acetylcholine receptors
- Pernicious anemia-Gastric parietal cells; intrinsic factor
- Hashimoto's thyroiditis-Thyroid proteins and cells
- Autoimmune hemolytic anemia-RBC membrane proteins
- Spontaneous infertility-Sperm.

Systemic Autoimmune Diseases

- Multiple sclerosis-Brain or white matter
- Rheumatoid arthritis-Connective tissue, IgG
- Scleroderma-Nuclei, heart, lungs, gastrointestinal tract, kidney
- Sjogren's syndrome-Salivary gland, liver, kidney, thyroid
- Systemic lupus erythematosus (SLE)- DNA, nuclear protein, RBC and platelet membranes

Treatment

- Iimmunosuppressive drugs.
- Thymectomy.
- Plasmapheresis for diseases involving immune complexes.
- vaccination with T cells specific for a given autoantigen
- Administration of synthetic blocking peptides that compete with autoantigen for binding to MHC molecules
- Treatment with monoclonal antibodies
- Induction of tolerance

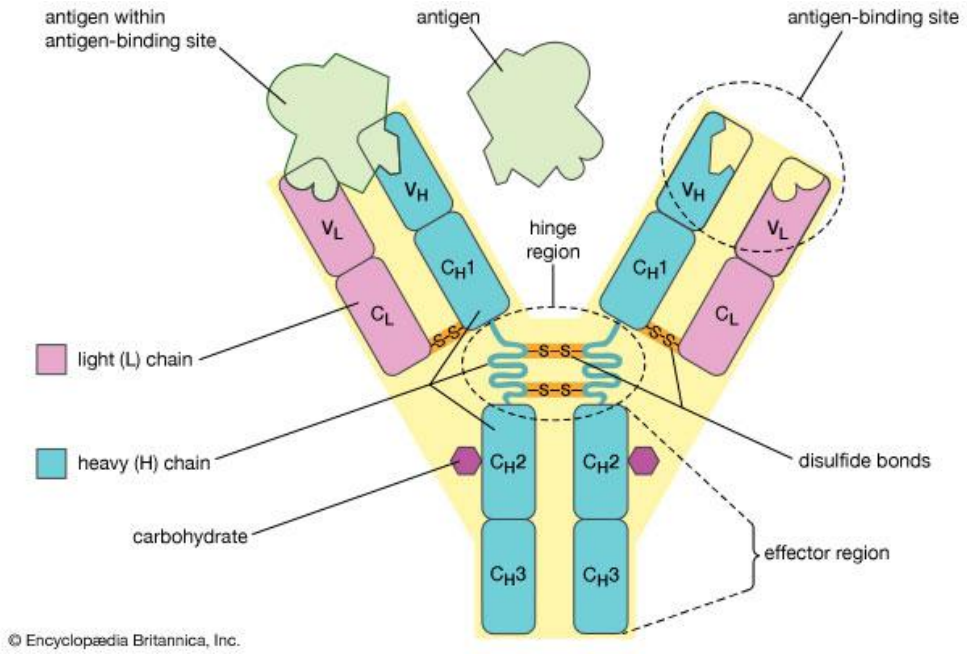
Unit –IV- Immunoglobulin and Vaccine

Immunoglobulin: Structure and Functions

- **Immunoglobulin (Ig):** Immunoglobulins are glycoprotein molecules that are produced by plasma cells in response to an immunogen and which function as antibodies. The immunoglobulins derive their name from the finding that they migrate with globular proteins when antibody-containing serum is placed in an electrical field.

Basic structure of Immunoglobulin: Although different immunoglobulins can differ structurally, they all are built from the same basic units.

- **Heavy and Light Chains**
    - All immunoglobulins have a four chain structure as their basic unit. They are composed of two identical light chains (23kD) and two identical heavy chains (50-70kD)
- **Disulfide bonds**
    - **Inter-chain disulfide bonds** - The heavy and light chains and the two heavy chains are held together by inter-chain disulfide bonds and by non-covalent interactions The number of inter-chain disulfide bonds varies among different immunoglobulin molecules.
    - **Intra-chain disulfide binds** - Within each of the polypeptide chains there are also intra-chain disulfide bonds.
- **Variable (V) and Constant (C) Regions**
    - When the amino acid sequences of many different heavy chains and light chains were compared, it became clear that both the heavy and light chain could be divided into two regions based on variability in the amino acid sequences. These are the:
        - **Light Chain** - VL (110 amino acids) and CL (110 amino acids)
        - **Heavy Chain** - VH (110 amino acids) and CH (330-440 amino acids)



- **Hinge Region**
  - This is the region at which the arms of the antibody molecule forms a Y. It is called the hinge region because there is some flexibility in the molecule at this point.
- **Domains**
  - Rather, it is folded into globular regions each of which contains an intra-chain disulfide bond. These regions are called domains.
    - **Light Chain Domains** - VL and CL
    - **Heavy Chain Domains** - VH, CH1 - CH3 (or CH4)
- **Oligosaccharides**
  - Carbohydrates are attached to the CH2 domain in most immunoglobulins. However, in some cases carbohydrates may also be attached at other locations.

Antigen-binding site

- • Antigen binding occurs at 3 HYPERVARIABLE regions, known as COMPLEMENTARITY DETERMINING REGIONS (CDR's)

- • These have specific residue positron numbers
- • The region of binding is a large undulating 3D structure (~750A = 10-10m), so is highly specific and there are a significant number of interactions between the antibody and antigen surface

Forces involved

- • Hydrogen bonds
- • Ionic bonds
- • Hydrophobic interactions
- • Van der Waals interactions

Antibody Affinity

- The strength of the total non-covalent interactions between a **single antigen binding site** and a **single epitope** on the antigen.
- The affinity association constant K can be calculated:
- K varies from 104 to 1011 L/mol

Antibody Avidity

- The overall strength of **multiple interactions** between an antibody with **multiple binding sites** and a complex antigen with **multiple epitopes**
- • This is a better measure of binding capacity in biological systems
- • **Monovalent** interactions have a low affinity
- • **Bivalent** interactions have a high affinity
- • **Polyvalent** interactions have a very high affinity

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Cross-Reactivity

- Antibodies elicited in response to one antigen can also recognise a different antigen, for example:

  1. Vaccination with cowpox induces antibodies which are able to recognise smallpox

  2. ABO blood group antigens are glycoproteins on red blood cells. Antibodies made against microbial agents on common intestinal bacteria may cross-react with the glycoproteins, which poses a problem for blood transfusions.

Immunoglobulin Fragments: Structure and function relationship

Immunoglobulin fragments produced by proteolytic digestion have proven very useful in elucidating structure/function relationships in immunoglobulins.

- **Fab - fraction antigen binding:** this is the highly variable area which determines specificity
- **Fc - fraction crystalline:** which determines what happens once the Ab-Ag interaction has occurred
- **F(ab')2**

Human immunoglobulin

A. Immunoglobulin classes: The immunoglobulins can be divided into five different classes, based on differences in the amino acid sequences in the constant region of the heavy chains. All immunoglobulins within a given class will have very similar heavy chain constant regions. These differences can be detected by sequence studies or more commonly by serological means (i.e. by the use of antibodies directed to these differences).

1. IgG - Gamma heavy chains
2. IgM - Mu heavy chains
3. IgA - Alpha heavy chains

4. IgD - Delta heavy chains
5. IgE - Epsilon heavy chains

Structure and some properties of IG classes and subclasses

IgG

- **Structure**
    - All IgG's are monomers (7S immunoglobulin). The subclasses differ in the number of disulfide bonds and length of the hinge region.
- **Properties**
- IgG is the most versatile immunoglobulin because it is capable of carrying out all of the functions of immunoglobulin molecules.

    - IgG is the major Ig in serum - 75% of serum Ig is IgG
    - IgG is the major Ig in extra vascular spaces
    - Placental transfer - IgG is the only class of Ig that crosses the placenta. Transfer is mediated by a receptor on placental cells for the Fc region of IgG. Not all subclasses cross equally well; IgG2 does not cross well.
    - Fixes complement - Not all subclasses fix equally well; IgG4 does not fix complement
    - Binding to cells - Macrophages, monocytes, PMNs and some lymphocytes have Fc receptors for the Fc region of IgG. Not all subclasses bind equally well; IgG2 and IgG4 do not bind to Fc receptors. A consequence of binding to the Fc receptors on PMNs, monocytes and macrophages is that the cell can now internalize the antigen better. The antibody has prepared the antigen for eating by the phagocytic cells. The term opsonin is used to describe substances that enhance phagocytosis.

IgM

- **Structure**

- IgM normally exists as a pentamer (19S immunoglobulin) but it can also exist as a monomer. In the pentameric form all heavy chains are identical and all light chains are identical. Thus, the valence is theoretically 10. IgM has an extra domain on the mu chain (CH4) and it has another protein covalently bound via a S-S bond called the J chain. This chain functions in polymerization of the molecule into a pentamer.

- **Properties**

  - IgM is the third most common serum Ig.
  - IgM is the first Ig to be made by the fetus and the first Ig to be made by a virgin B cells when it is stimulated by antigen.
  - As a consequence of its pentameric structure, IgM is a good complement fixing Ig. Thus, IgM antibodies are very efficient in leading to the lysis of microorganisms.
  - As a consequence of its structure, IgM is also a good agglutinating Ig . Thus, IgM antibodies are very good in clumping microorganisms for eventual elimination from the body.
  - IgM binds to some cells via Fc receptors.
  - B cell surface Ig
  - Surface IgM exists as a monomer and lacks J chain but it has an extra 20 amino acids at the C-terminus to anchor it into the membrane. Cell surface IgM functions as a receptor for antigen on B cells. Surface IgM is noncovalently associated with two additional proteins in the membrane of the B cell called Ig-alpha and Ig-beta. These additional proteins act as signal transducing molecules since the cytoplasmic tail of the Ig molecule itself is too short to transduce a signal. Contact between surface immunoglobulin and an antigen is required before a signal can be transduced by the Ig-alpha and Ig-beta chains. In the case of T-independent antigens, contact between the antigen and surface immunoglobulin is sufficient to activate B cells to differentiate into antibody secreting plasma cells. However, for T-dependent antigens, a second signal provided by helper T cells is required before B cells are activated.

IgA

- **Structure**
  - When IgA exits as a dimer, a J chain is associated with it.
  - When IgA is found in secretions is also has another protein associated with it called the secretory piece or T piece; sIgA is sometimes referred to as 11S immunoglobulin. Unlike the remainder of the IgA which is made in the plasma cell, the secretory piece is made in epithelial cells and is added to the IgA as it passes into the secretions. The secretory piece helps IgA to be transported across mucosa and also protects it from degradation in the secretions.
- **Properties**
  - IgA is the 2nd most common serum Ig.
  - IgA is the major class of Ig in secretions - tears, saliva, colostrum, mucus. Since it is found in secretions secretory IgA is important in local (mucosal) immunity.
  - Normally IgA does not fix complement, unless aggregated.
  - IgA can binding to some cells - PMN's and some lymphocytes.

IgD

- **Structure**
  - IgD exists only as a monomer.
- **Properties**
  - IgD is found in low levels in serum; its role in serum uncertain.
  - IgD is primarily found on B cell surfaces where it functions as a receptor for antigen. IgD on the surface of B cells has extra amino acids at C-terminal end for anchoring to the membrane. It also associates with the Ig-alpha and Ig-beta chains.
  - IgD does not bind complement.

IgE

- **Structure**

  IgE exists as a monomer and has an extra domain in the constant region.
- **Properties**
  - IgE is the least common serum Ig since it binds very tightly to Fc receptors on basophils and mast cells even before interacting with antigen.
  - Involved in allergic reactions - As a consequence of its binding to basophils an mast cells, IgE is involved in allergic reactions. Binding of the allergen to the IgE on the cells results in the release of various pharmacological mediators that result in allergic symptoms.
  - IgE also plays a role in parasitic helminth diseases. Since serum IgE levels rise in parasitic diseases, measuring IgE levels is helpful in diagnosing parasitic infections. Eosinophils have Fc receptors for IgE and binding of eosinophils to IgE-coated helminths results in killing of the parasite.
  - IgE does not fix complement.

Clinical implications of human immunoglobulin classes

IgG

- **Increases in:** a) Chronic granulomatous infections; b) Infections of all types; c) Hyperimmunization; d) Liver disease; e) Malnutrition (severe); f) Dysproteinemia; g) Disease associated with hypersensitivity granulomas, dermatologic disorders, and IgG myeloma; h) Rheumatoid arthritis
- **Decreases in:** a) Agammaglobulinemia; b) Lymphoid aplasia; c) Selective IgG, IgA deficiency; d) IgA myeloma e) Bence Jones proteinemia; f) Chronic lymphoblastic leukemia

IgM

- **Increases (in adults) in:** a) Waldenström's macroglobulinemia; b) Trypanosomiasis; c) Actinomycosis; d) Carrión's disease (bartonellosis); e) Malaria; f) Infectious mononucleosis; g)

Lupus erythematosus; h) Rheumatoid arthritis; I) Dysgammaglobulinemia (certain cases).

- **Note:** In the newborn, a level of IgM above 20 ng./dl is an indication of in utero stimulation of the immune system and stimulation by the rubella virus, the cytomegalovirus, syphilis, or toxoplasmosis.
- **Decreases in:** a) Agammaglobulinemia; b) Lymphoproliferative disorders (certain cases); c) Lymphoid aplasia; d) IgG and IgA myeloma; e) Dysgammaglobulinemia; f) Chronic lymphoblastic leukemia

IgA

- **Increases in:** a) Wiskott-Aldrich syndrome; b) Cirrhosis of the liver (most cases); c) Certain stages of collagen and other autoimmune disorders such as rheumatoid arthritis and lupus erythematosus; d) Chronic infections not based on immunologic deficiencies; e) IgA myeloma
- **Decreases in:** a) Hereditary ataxia telangiectasia; b) Immunologic deficiency states (e.g., dysgammaglobulinemia, congenital and acquired agammaglobulinemia, and hypogammaglobulinemia); c) Malabsorption syndromes; d) Lymphoid aplasia; e) IgG myeloma; f) Acute lymphoblastic leukemia; g) Chronic lymphoblastic leukemia

IgD

1. Increases in: a) Chronic infections; b) IgD myelomas

IgE

- **Increases in:** a) Atopic skin diseases such as eczema; b) Hay fever; c) Asthma; d) Anaphylactic shock; e) IgE-myeloma
- **Decreases in:** a) Congenital agammaglobulinemia; b) Hypogammaglobulinemia due to faulty metabolism or synthesis of immunoglobulins

Selective Immunoglobulin Distribution

- • IgG and IgM in blood
- • IgG in extracellular fluid
- • Dimeric IgA in secretions across epithelia, including breast milk
- • Maternal IgG in foetus via placental transfer
- • IgE with mast cells below epithelium
    - • Brain devoid of antibodies

Isotypes and Allotypes

• Isotypes are antibodies who are present in everybody, with a constant region.

• Allotypes are antibodies that contain single amino acid mutations, giving allelic polymorphisms which vary in the population

Humoral and cell-mediated immunity defend against different types of threats

- The immune system can mount two types of responses to antigens: a humoral response and a cell-mediated response.
- **Humoral immunity** involves B cell activation and clonal selection and results in the production of antibodies that circulate in the blood plasma and lymph.
- Circulating antibodies defend mainly against free bacteria, toxins, and viruses in the body fluids.
- In **cell-mediated immunity,** activation and clonal selection of cytotoxic T lymphocytes allows these cells to directly destroy certain target cells, including "nonself" cancer and transplant cells.
- The humoral and cell-mediated immune responses are linked by cell-signaling interactions, especially via **helper T cells.**

Helper T lymphocytes function in both humoral and cell-mediated immunity.

- When a helper T cell recognizes a class II MHC molecule-antigen complex on an antigen-presenting cell, the helper T cell proliferates and differentiates into a clone of activated helper T cells and memory helper T cells.
- A surface protein called CD4 binds the side of the class II MHC molecule.
- This interaction helps keep the helper T cell and the antigen-presenting cell joined while activation of the helper T cell proceeds.
- Activated helper T cells secrete several different cytokines that stimulate other lymphocytes, thereby promoting cell-mediated and humoral responses.
- Dendritic cells are important in triggering a primary immune response.
    - They capture antigens, migrate to the lymphoid tissues, and present antigens, via class II MHC molecules, to helper T cells.
- Macrophages present antigens to memory helper T cells, while B cells primarily present antigens to helper T cells in the course of the humoral response.

In the cell-mediated response, cytotoxic T cells counter intracellular pathogens.

- Antigen-activated cytotoxic T lymphocytes kill cancer cells and cells infected by viruses and other intracellular pathogens.
- Fragments of nonself proteins synthesized in such target cells associate with class I MHC molecules and are displayed on the cell surface, where they can be recognized by cytotoxic T cells.
    - This interaction is greatly enhanced by the T surface protein **CD8** that helps keep the cells together while the cytotoxic T cell is activated.
- When a cytotoxic T cell is activated by specific contacts with class I MHC-antigen complexes on an infected cell, the activated cytotoxic T cell differentiates into an active killer, which kills its target cell—the antigen-presenting cell—primarily by secreting proteins that act on the bound cell.
    - The death of the infected cell not only deprives the pathogen of a place to reproduce, but also exposes it to circulating antibodies, which mark it for disposal.

- Once activated, cytotoxic T cells kill other cells infected with the same pathogen.

- In the same way, cytotoxic T cells defend against malignant tumors.
  - Because tumor cells carry distinctive molecules not found on normal cells, they are identified as foreign by the immune system.
  - Class I MHC molecules on a tumor cell present fragments of tumor antigens to cytotoxic T cells.
  - Interestingly, certain cancers and viruses actively reduce the amount of class I MHC protein on affected cells so that they escape detection by cytotoxic T cells.
  - The body has a backup defense in the form of natural killer cells, part of the nonspecific defenses, which lyse virus-infected and cancer cells.

In the humoral response, B cells make antibodies against extracellular pathogens.

- Antigens that elicit a humoral immune response are typically proteins and polysaccharides present on the surface of bacteria or transplanted tissue.
- The activation of B cells is aided by cytokines secreted by helper T cells activated by the same antigen.
  - These B cells proliferate and differentiate into a clone of antibody-secreting plasma cells and a clone of memory B cells.
- When antigen first binds to receptors on the surface of a B cell, the cell takes in a few of the foreign molecules by receptor-mediated endocytosis.
- The B cell then presents antigen fragments to a helper B cell.
- Many antigens (primarily proteins), called **T-dependent antigens,** can trigger a humoral immune response by B cells only with the participation of helper T cells.
- Other antigens, such as polysaccharides and proteins with many identical polypeptides, function as **T-independent antigens.**
  - These include the polysaccharides of many bacterial capsules and the proteins of the bacterial flagella.

- These antigens bind simultaneously to a number of membrane antibodies on the B cell surface.
- This stimulates the B cell to generate antibody-secreting plasma cells without the help of cytokines.
- While this response is an important defense against many bacteria, it generates a weaker response than T-dependent antigens and generates no memory cells.

- Any given humoral response stimulates a variety of different B cells, with each giving rise to a clone of thousands of plasma cells.
  - Each plasma cell is estimated to secrete about 2,000 antibody molecules per second over the cell's 4- to 5-day life span.
  - A secreted antibody has the same general Y-shaped structure as a B cell receptor, but lacks a transmembrane region that would anchor it to a plasma membrane.
- Antigens that elicit a humoral immune response are typically the protein and polysaccharide surface components of microbes, incompatible transplanted tissues, or incompatible transfused cells.
  - In addition, for some humans, the proteins of foreign substances such as pollen or bee venom act as antigens that induce an allergic, or hypersensitive, humoral response.
- Antibodies constitute a group of globular serum proteins called **immunoglobins (Igs).**
- There are five major types of heavy-chain constant regions, determining the five major classes of antibodies.
  - Two classes exist primarily as polymers of the basic antibody molecule: IgM as a pentamer and IgA as a dimmer.
  - The other three classes—IgG, IgE, and IgD—exist exclusively as monomers,
- The power of antibody specificity and antigen-antibody binding has been applied in laboratory research, clinical diagnosis, and disease treatment.
  - Some antibody tools are polyclonal, the products of many different clones of B cells, each specific for a different epitope.
  - Others are monoclonal, prepared from a single clone of B cells grown in culture.

        - These cells produce **monoclonal antibodies,** specific for the same epitope on an antigen.
        - These have been used to tag specific molecules.
        - For example, toxin-linked antibodies search and destroy tumor cells.

- The binding of antibodies to antigens is also the basis of several antigen disposal mechanisms.
    - In **viral neutralization,** antibodies bind to proteins on the surface of a virus, blocking the virus's ability to infect a host cell.
    - In **opsonization,** the bound antibodies enhance macrophage attachment to and phagocytosis of the microbes. Neither the B cell receptor for an antigen nor the secreted antibody actually binds to an entire antigen molecule.
- Antibody-mediated **agglutination** of bacteria or viruses effectively neutralizes and opsonizes the microbes.
    - Agglutination is possible because each antibody molecule has at least two antigen-binding sites.
    - IgM can link together five or more viruses or bacteria.
    - These large complexes are readily phagocytosed by macrophages.
- In **precipitation,** the cross-linking of soluble antigen molecules—molecules dissolved in body fluids—forms immobile precipitates that are disposed of by phagocytosis.
- The **complement system** participates in the antibody-mediated disposal of microbes and transplanted body cells.
- The pathway begins when IgM or IgG antibodies bind to a pathogen, such as a bacterium.
    - The first complement component links two bound antibodies and is activated, initiating the cascade.
        - Ultimately, complement proteins generate a **membrane attack complex (MAC),** which forms a pore in the bacterial membrane, resulting in cell lysis.

- Whether activated as part of innate or acquired defenses, the complement cascade results in the lysis of microbes and produces activated complement proteins that promote inflammation or stimulate phagocytosis.

Vaccine

Immunity conferred by recovering from an infectious disease such as chicken pox is called active immunity **because it depends on the response of the infected person's own immune system.**

- Active immunity can be acquired naturally or artificially, by **immunization,** also known as **vaccination.**
- Vaccines include inactivated bacterial toxins, killed microbes, parts of microbes, viable but weakened microbes, and even genes encoding microbial proteins.
- These agents can act as antigens, stimulating an immune response and, more important, producing immunological memory.

- A vaccinated person who encounters the actual pathogen will have the same quick secondary response based on memory cells as a person who has had the disease.
  - Routine immunization of infants and children has dramatically reduced the incidence of infectious diseases such as measles and whooping cough, and has led to the eradication of smallpox, a viral disease.
  - Unfortunately, not all infectious agents are easily managed by vaccination.
    - For example, the emergence of new strains of pathogens with slightly altered surface antigens complicates development of vaccines against some microbes, such as the parasite that causes malaria.
- Antibodies can be transferred from one individual to another, providing **passive immunity.**
  - This occurs naturally when IgG antibodies of a pregnant woman cross the placenta to her fetus.
  - In addition, IgA antibodies are passed from mother to nursing infant in breast milk.
  - Passive immunity persists as long as these antibodies last, a few weeks to a few months.

- This protects the infant from infections until the baby's own immune system has matured.

- Passive immunity can be transferred artificially by injecting antibodies from an animal that is already immune to a disease into another animal.
  - This confers short-term, but immediate, protection against that disease.
  - For example, a person bitten by a rabid animal may be injected with antibodies against rabies virus because rabies may progress rapidly, and the response to an active immunization could take too long to save the life of the victim.
    - Most people infected with rabies virus are given both passive immunizations (the immediate defense) and active immunizations (a longer-term defense).

Vaccine-Preventable Diseases and the Vaccines that Prevent Them

Disease	Vaccine	Disease spread by	Disease symptoms	Disease complications
Chickenpox	Varicella vaccine protects against chickenpox.	Air, direct contact	Rash, tiredness, headache, fever	Infected blisters, bleeding disorders, encephalitis (brain swelling), pneumonia (infection in the lungs)
Diphtheria	DTaP* vaccine protects against diphtheria.	Air, direct contact	Sore throat, mild fever, weakness, swollen glands in neck	Swelling of the heart muscle, heart failure, coma, paralysis, death
Hib	Hib vaccine protects against *Haemophilus influenzae* type b.	Air, direct contact	May be no symptoms unless bacteria enter the blood	Meningitis (infection of the covering around the brain and spinal cord), intellectual disability, epiglottis (life-threatening infection that can block the windpipe and lead to serious breathing problems), pneumonia (infection in the lungs), death
Hepatitis A	HepA vaccine protects against hepatitis A.	Direct contact, contaminated food or water	May be no symptoms, fever, stomach pain, loss of appetite, fatigue, vomiting, jaundice (yellowing of skin and eyes), dark urine	Liver failure, arthralgia (joint pain), kidney, pancreatic, and blood disorders
Hepatitis B	HepB vaccine protects against hepatitis B.	Contact with blood or body fluids	May be no symptoms, fever, headache, weakness, vomiting, jaundice (yellowing of skin and eyes), joint pain	Chronic liver infection, liver failure, liver cancer
Flu	Flu vaccine protects against influenza.	Air, direct contact	Fever, muscle pain, sore throat, cough, extreme fatigue	Pneumonia (infection in the lungs)
Measles	MMR** vaccine protects against measles.	Air, direct contact	Rash, fever, cough, runny nose, pinkeye	Encephalitis (brain swelling), pneumonia (infection in the lungs), death
Mumps	MMR**vaccine protects against mumps.	Air, direct contact	Swollen salivary glands (under the jaw), fever, headache, tiredness, muscle pain	Meningitis (infection of the covering around the brain and spinal cord) , encephalitis (brain swelling), inflammation of testicles or ovaries, deafness
Pertussis	DTaP* vaccine protects against pertussis (whooping cough).	Air, direct contact	Severe cough, runny nose, apnea (a pause in breathing in infants)	Pneumonia (infection in the lungs), death
Polio	IPV vaccine protects against polio.	Air, direct contact, through the mouth	May be no symptoms, sore throat, fever, nausea, headache	Paralysis, death
Pneumococcal	PCV vaccine protects against pneumococcus.	Air, direct contact	May be no symptoms, pneumonia (infection in the lungs)	Bacteremia (blood infection), meningitis (infection of the covering around the brain and spinal cord), death
Rotavirus	RV vaccine protects against rotavirus.	Through the mouth	Diarrhea, fever, vomiting	Severe diarrhea, dehydration
Rubella	MMR** vaccine protects against rubella.	Air, direct contact	Children infected with rubella virus sometimes have a rash, fever, swollen lymph nodes	Very serious in pregnant women—can lead to miscarriage, stillbirth, premature delivery, birth defects
Tetanus	DTaP* vaccine protects against tetanus.	Exposure through cuts in skin	Stiffness in neck and abdominal muscles, difficulty swallowing, muscle spasms, fever	Broken bones, breathing difficulty, death

* DTaP combines protection against diphtheria, tetanus, and pertussis.
** MMR combines protection against measles, mumps, and rubella.

Last updated on 03/20/2013 • CS239274-A

Adjuvants

- Substances that can enhance the immune response to an immunogen (antigen) are called adjuvants. They are not antigenic by themselves and alone cannot initiate any immune response by themselves. Adjuvants have the capability of stimulating the rapid and sustained production of high titers of antibodies with high avidity and influencing some properties of cell-mediated immunity.

The action of adjuvants include

- Enhance long-term release of the antigen by functioning as a depot.
- Increases the number of cells involved in immune response.
- Adjuvants may act as non-specific mediators of immune cell function by stimulating or modulating immune cells.
- Provides more efficient processing of antigen.
- Adjuvants may also enhance macrophage phagocytosis after binding the antigen.
- Increase the rate of synthesis and release of antibodies.
- Stimulates the production of cytokines

Types of adjuvants

- **Repository adjuvants:** These include aluminum (aluminum hydroxide, aluminum phosphate) and calcium (calcium alginate) salts.
- **Water-in-oil emulsions:** The emulsified adjuvant allows slow release of antigen from oil droplets. Since there is variety in droplet size, the droplets are degraded at different rates resulting in prolonged presence of antigen.
- **Nitrocellulose-adsorbed antigen:** The nitrocellulose is basically inert, leading to almost no inflammatory response.
- **Lipopolysaccharide (endotoxin):** Endotoxins of gram negative bacteria may be used as adjuvant.

- **Bordetella pertussis:** This bacterium is another adjuvant that increases the amount of IgM produced.
- **Immune-stimulating complexes** (ISCOMs) are antigen modified saponin/cholesterol micelles. **Liposomes** can also be used as they are based on similar principle.
- **BCG:** Nonspecific immune stimulation of cancer patients with adjuvants such as BCG has been tried for many years.
- **L-tyrosine:** A co-precipitate of the amino acid and antigen has been shown to have excellent adjuvant properties, even surpassing Freund's complete adjuvant in some circumstances.

Allotypes

- Allotypes are antigenic determinants specified by allelic forms of the Ig genes.
- Allotypes represent slight differences in the amino acid sequences of heavy or light chains of different individuals. Even a single amino acid difference can give rise to an allotypic determinant, although in many cases there are several amino acid substitutions that have occurred.

Occurrence

- In man the allotypic differences are localized to the constant region of the heavy and light chains.
- Individual allotypes are found in individual members of a species. All allotypes are not found in all members of the species. The prefix Allo means different in individuals of a species

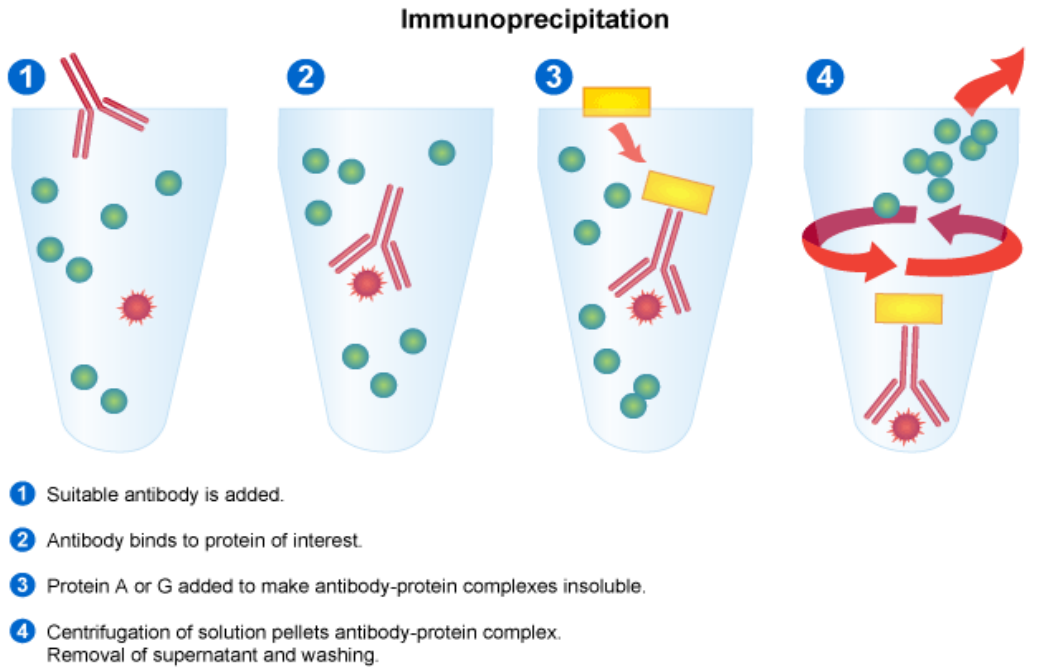
Importance

- **1. Monitoring bone marrow grafts** - Bone marrow grafts that produce a different allotype from the recipient can be used to monitor the graft.
- **2. Forensic medicine** - Km and Gm allotypes are detectable in blood stains and semen and are useful in forensic medicine.

- **3. Paternity testing** - The immunoglobulin allotypes are one of the characteristics used in legal cases involving paternity.

UNIT V-IMMUNOLOGICAL TECHNIQUES

Immunoprecipitation (IP) is the technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. This process can be used to isolate and concentrate a particular protein from a sample containing many thousands of different proteins. Immunoprecipitation requires that the antibody be coupled to a solid substrate at some point in the procedure.



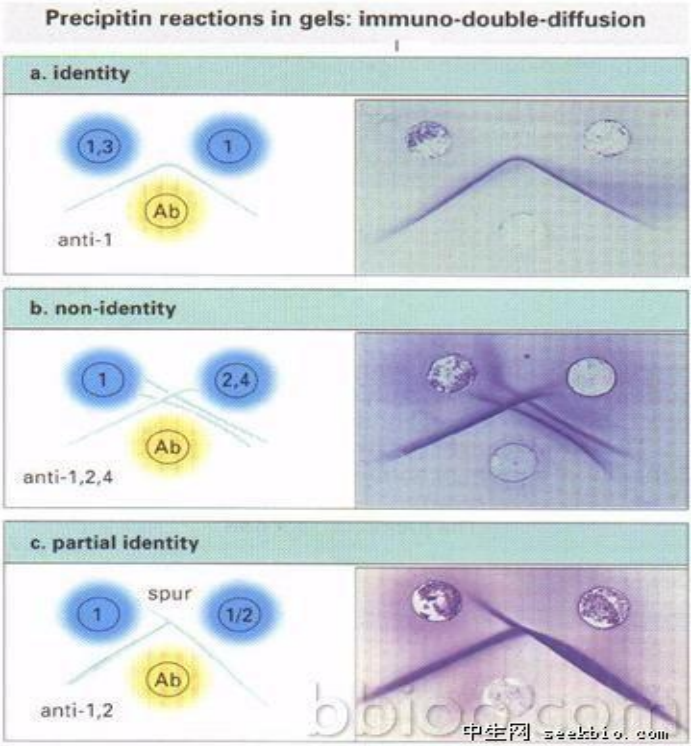
Immunodiffussion

Immunodiffusion refers to the movement of antigen or antibody or both antigen and antibody molecules in a support medium by diffusion.

Procedure

Molten agar is poured on glass slide or petridish and allowed to solidify. Two small wells at few millimeter distance apart are punched out. Antigen and corresponding antibody solutions are dropped in opposite wells. The slide/petridish is then kept in a moist chamber for 18-24 hours.

The antigen and antibody move through the agar and combine to form antigen-antibody complex. The antigen-antibody complex is visible as a precipitation line.



Reaction of identity:

Antigens in two wells are identical (Agl and Ag2). So the reaction with the antibody results in a precisely similar precipitin lines. The precipitin lines don't cross, but form a continuous line. Fusion of the two precipitin lines indicates that the antibody is reacting with epitopes commonly present on the two antigens.

ii. Reaction of non-identity:

Two non-identical antigens (Agl and Ag3) are in the antigen wells while the antibody well has antibodies to both the antigens. The two antigen-antibody precipitin lines formed differ from each other. Hence the two precipitin lines completely cross (intersect) each other.

iii. Reaction of partial identity:

The antigenic determinants in the antigens of two wells are partially shared (Agl and Agla). Hence the antibody reacts with both the antigens and forms lines that do not form a complete cross. The precipitin line crosses in only one direction. The extended precipitin line is referred to as a spur. The spur indicates that the antibody is also precipitating an additional epitope that is not present in one of the antigens.

Single Radial Immunodiffusion:

Either antigen or antibody remains fixed and the other reactant moves (Diffusion technique of Oudin).The antibody is mixed with molten agar and poured into a petri dish. After solidification of the agar, wells are cut, and filled with different concentrations of antigen .

The precipitate is seen as a white circular line around each well. The diameter of the circular ring is directly proportional to the antigen concentration (i.e., more the antigen concentration, the diameter of the precipitate is more).

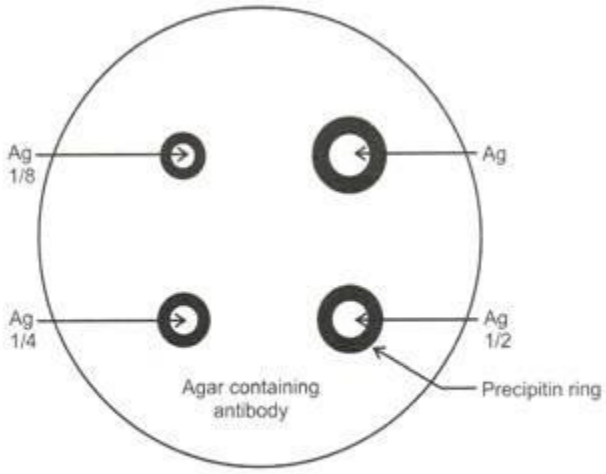
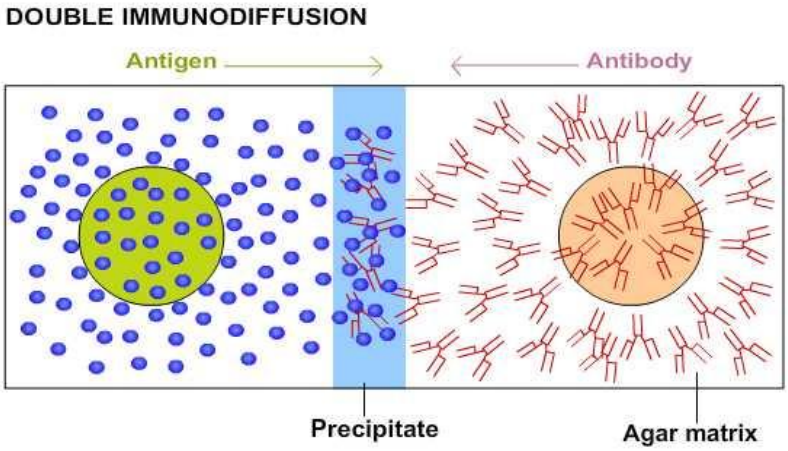


Fig. 27.4: Single radial immunodiffusion

A standard curve can be plotted in a graph sheet by plotting the known antigen concentrations in the X axis and the diameters in the Y axis. The test antigen, whose concentration is unknown, can be determined by interpolating the standard curve with the diameter of the precipitate formed by the test antigen.

Double immunodiffusion

Ouchterlony double immunodiffusion (also known as agar gel **immunodiffusion**or passive **double immunodiffusion**) is an immunological technique used in the detection, identification and quantification of antibodies and antigens, such as immunoglobulins and extractable nuclear antigens.



- The Ouchterlony double diffusion (ODD) technique is one of the simplest techniques extensively used to check antisera for the presence of antibodies for a particular Ag and to determine its titre.

- This method has been widely used for detection and qualitative diagnostic procedures. The method is called "double" referring to the fact that in this procedure, antigen and antibody are allowed to migrate towards each other in a gel and a line of precipitation is formed where the two reactants meet.

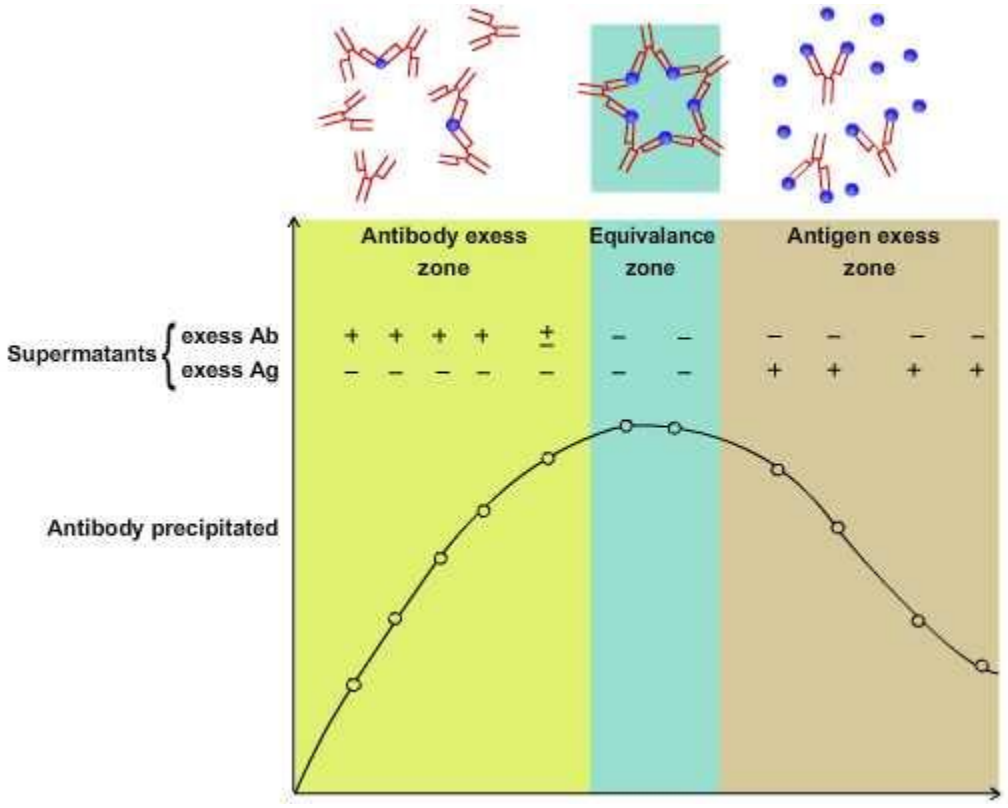
- This precipitation reaction is highly specific. The method is even today widespread and used by people working with diagnosis or protein detection or comparing antigens or antisera.

  The technique involves cutting wells into an Agarose solidified in a glass plate. The wells are filled with antibody or antigen and the plate is incubated. When homologous antigen and antibody diffuse toward each other from the individual wells, a precipitin line will form somewhere between the two wells.

- Precipitation occurs because the antigen is multivalent i.e,has several antigenic determinants per molecule to which antibodies can bind. Antibodies have at least two antigen binding sites, thus large aggregates or lattices of antigen and antibody are formed.

- Precipitation will not occur if excess antigen is present or if excess antibody is present. Cross-linking and lattice formation will only occur when antigen and antibody concentrations are optimal. An increasing amount of antigen is added to a constant amount of antibody in solution. This is called the antibody-excess zone (Prozone phenomenon). The Ag and Ab concentrations are relatively higher near their respective wells. As they diffuse farther from the wells, their concentration decreases. An antigen will react with its specific antibody to form an Ag-Ab complex. As more antigens are added, the amount of protein precipitated increases until the antigen/antibody molecules are at an optimal ratio. This is known as the equivalence

zone or equivalence point. When the amount of antigen in solution exceeds the amount of antibody, the amount of precipitation will decrease. This is known as the antigen excess zone.



Immunoelectrophoresis

Immunoelectrophoresis was first coined by Grabar and Williams in 1953. After electrophoretic separation of serum proteins in an agar gel, the serum proteins were allowed to diffuse against antibodies, leading to a pattern of precipitation arcs in the agar representing the major constituents among the serum proteins.

Immunoelectrophoresis is a general name for a number of biochemical methods for separation and characterization of proteins based on electrophoresis and reaction with antibodies. All variants of immunoelectrophoresis require immunoglobulins, also known as antibodies, reacting with the proteins to be separated or characterized.

Principle

On the basis of the difference in surface charge between the different protein molecules of antigens, several antigens can be separated from a mixture by electrophoresis in agar gel.

Procedure

Immunoelectrophoresis combines electrophoresis with immunodiffusion.

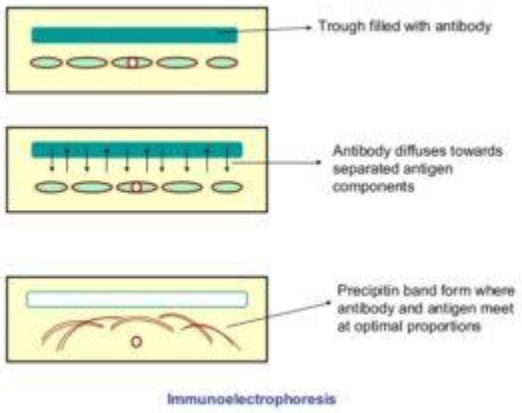
Stage 1

Antigen mixture is placed in a well cut agar gel on slide or plate. Electric current is passed through the agar for sometimes. Antigen migrate and get separated from each other according to their clearges.

Stage 2

A tough is cut in the gel parallel to the direction of migration of antigen and filled with antibody.

Allow to diffuse for some time. Antigen antibody diffuse and formed precipitation are where antibodies encounters the antigens in optional proportion.



Immunoelectrophoresis

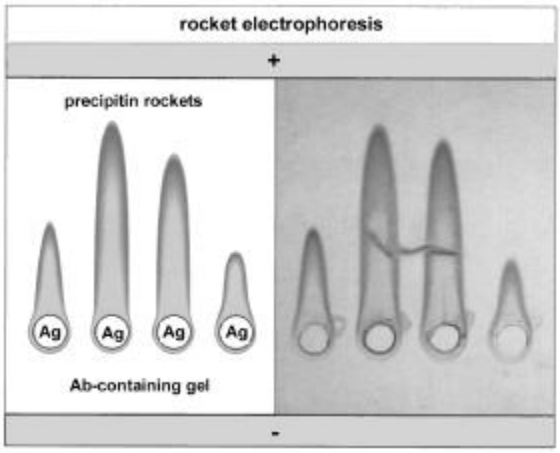
APPLICATIONS

- Useful in determination of presence and absence of serum protein.
- Example: Albumin,Immunoglobulin.

- Useful in detection of unuseful protein like human myeloma.
- Detect high antibody concentration.

Rocket immuno-electrophoresis

Rocket immuno-electrophoresis, a technique using antibodies, can also be utilized to detect allergenic proteins. The antibodies are contained in a gel, while samples are migrated through this gel by means of electrophoresis. Antigen-antibody complexes will form in the gel resulting in rocket-shaped precipitates. The formation of such complexes will only take place at a constant antigen/antibody ratio. Therefore, the height of the rocket is proportional to the amount of antigen in the sample.



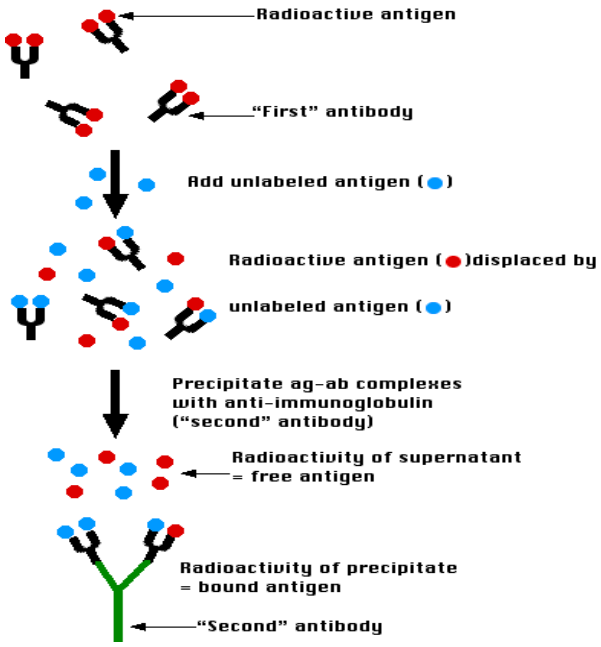
- This is a qualitative method.
- Antigen antibody complexes precipitate to form cone like structure (look like a rocket)
- Length of cone indicator the concentration of antigen.
- Negatively charged antigen are need for electrophoretic movement within agar matrix.

Radioimmunoassay(RIA)

Radioimmunoassay (**RIA**) is a very sensitive in vitro assay technique used to measure concentrations of antigens by use of antibodies.

- The labeled antigen is mixed with antibody at a concentration that saturates the antigen-binding sites of the antibody.
- Then test samples of unlabeled antigen of unknown concentration are added in progressively larger amounts.

- The antibody does not distinguish labeled from unlabeled antigen, so the two kinds of antigen compete for available binding sites on the antibody. As the concentration of unlabeled antigen increases, more labeled antigen will be displaced from the binding sites.
- The decrease in the amount of radiolabeled antigen bound to specific antibody in the presence of the test sample is measured in order to determine the amount of antigen present in the test sample.
- The antigen is generally labeled with a gamma-emitting isotope such as I^{125}, but beta-emitting isotopes such as tritium (3H) are also routinely used as labels.
- The radiolabeled antigen is part of the assay mixture; the test sample may be a complex mixture, such as serum or other body fluids, that contains the unlabeled antigen.

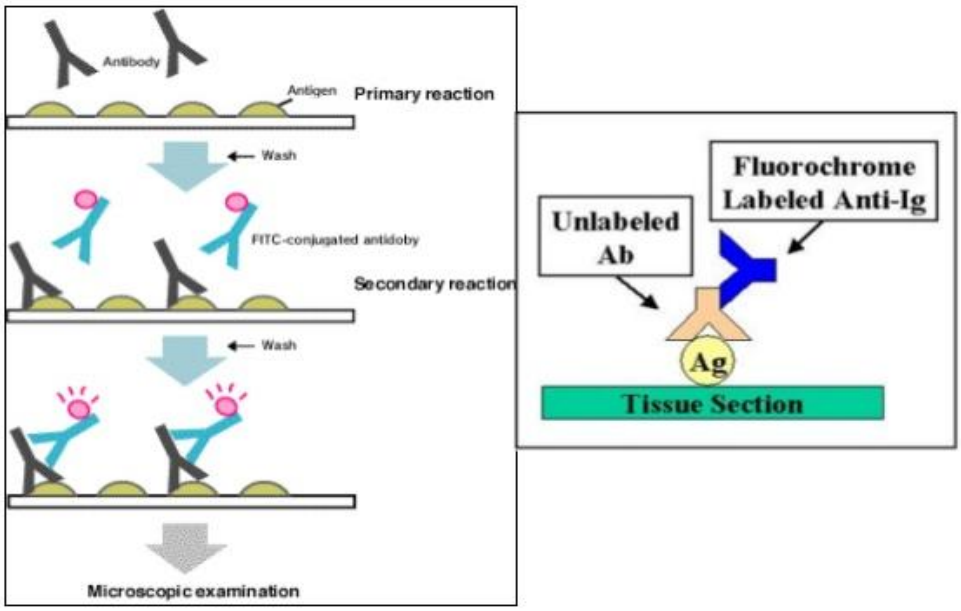


Application:

RIA has many uses, including narcotics (drug) detection, blood bank screening for the hepatitis (a highly contagious condition) virus, early cancer detection, measurement of growth hormone levels, tracking of the leukemia virus, diagnosis and treatment of peptic ulcers, and research with brain chemicals called neurotransmitters.

Immunofluorescence

- a technique used for the rapid identification of an antigen by exposing it to known antibodies tagged with the fluorescent dye fluorescein and observing the characteristic antigen-antibody reaction of precipitation.
- Immunofluorescence is a powerful technique that utilizes fluorescent-labeled antibodies to detect specific target antigens.
- The antibodies are chemically conjugated to fluorescent dyes such as fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC).
- These labeled antibodies bind (directly or indirectly) to the antigen of interest which allows for antigen detection through fluorescence techniques.
- There are two major types of immunofluorescence techniques, both based on the antigen and antibody reaction, in which the antibody attaches itself to a specific antigen.
- Techniques including direct immunofluorescence and indirect immunofluorescence.
- **Direct immunofluorescence** uses fluorescent-tagged antibodies to bind directly to the target antigen in the skin.
- **The indirect immunofluorescence** is used to detect circulating autoantibodies in immunobullous diseases.



Application:

- Immunofluorescence can be used on tissue sections, cultured cell lines, or individual cells, and may be used to analyse the distribution of proteins, glycans, and small biological and non-biological molecules.
- Direct immunofluorescence -This technique can be used to detect viral, parasitic, tumor antigens from patient specimens or monolayer of cells.
- Indirect immunofluorescence.-It is often used to detect autoantibodies. Commonly used in the detection of anti-nuclear antibodies (ANA) found in the serum of patients with SLE

Immunoblotting

- Immunoblotting techniques use antibodies (or other specific ligands in related techniques) to identify target proteins among a number of unrelated protein species
- They involve identification of protein target via antigen-antibody (or protein-ligand) specific reactions.
- Proteins are typically separated by electrophoresis and transferred onto membranes (usually nitrocellulose).
- The membrane is overlaid with a primary antibody for a specific target and then with a secondary antibody labeled, for example, with enzymes or with radioisotopes.

- When the ligand is not an antibody, the reaction can be visualized using a ligand that is directly labeled. Dot blot is a simplified procedure in which protein samples are not separated by electrophoresis but are spotted directly onto membrane.

Application

- immunoaffinity identification of proteins and analysis of immune responses
- genome-proteome interface technique.

ELISA

- **Enzyme-linked immunosorbent assay,** commonly known as **ELISA** (or EIA), is similar in principle to RIA but depends on an enzyme rather than a radioactive label
- The enzyme-linked immunosorbent assay (ELISA) depends on an enzyme-substrate reaction that generates a colored reaction product.
- A number of enzymes have been employed for ELISA, including alkaline phosphatase, horseradish peroxidase, and galactosidase.
    - Indirect ELISA
    - Sandwich ELISA
    - Competitive ELISA

Indirect ELISA

- sample containing primary antibody (Ab1) is added to an antigen- coated microtiter well and allowed to react with the antigen attached to the well.
- Free Ab1 is washed away, the presence of antibody bound to the antigen is detected by adding an enzyme-conjugated secondary anti-isotype antibody (Ab2), which binds to the primary antibody.

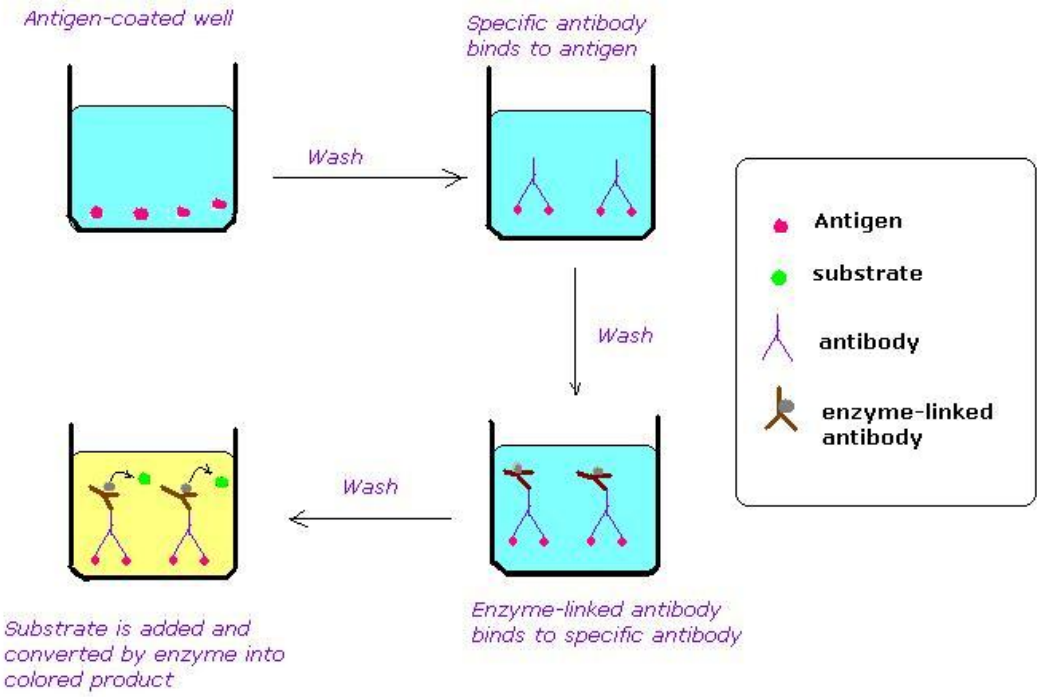
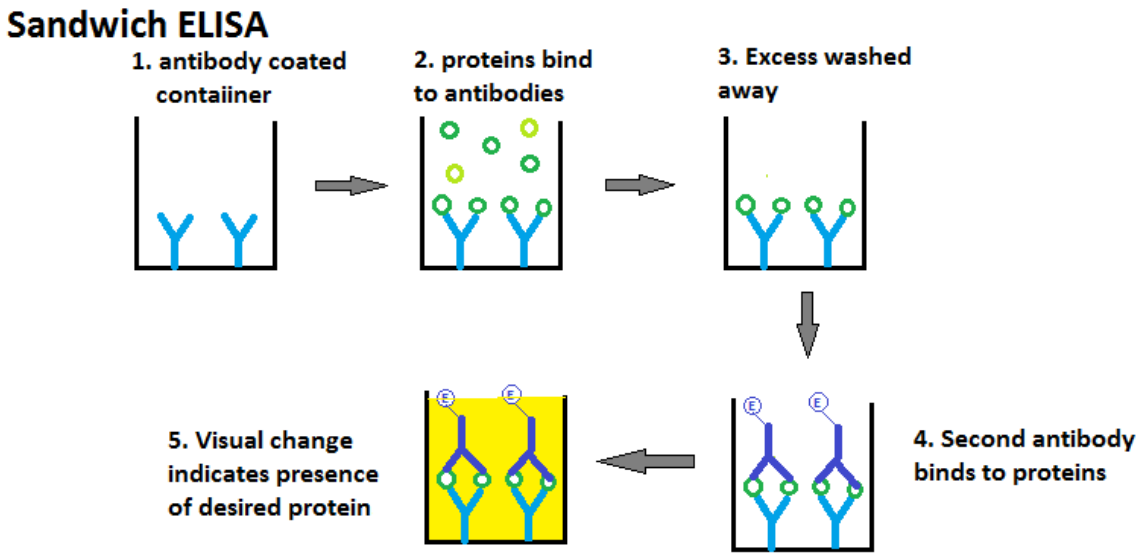


Figure 1: **Indirect ELISA**

- Any free Ab2 then is washed away, and a substrate for the enzyme is added. The amount of colored reaction product that forms is measured by specialized spectrophotometric plate .

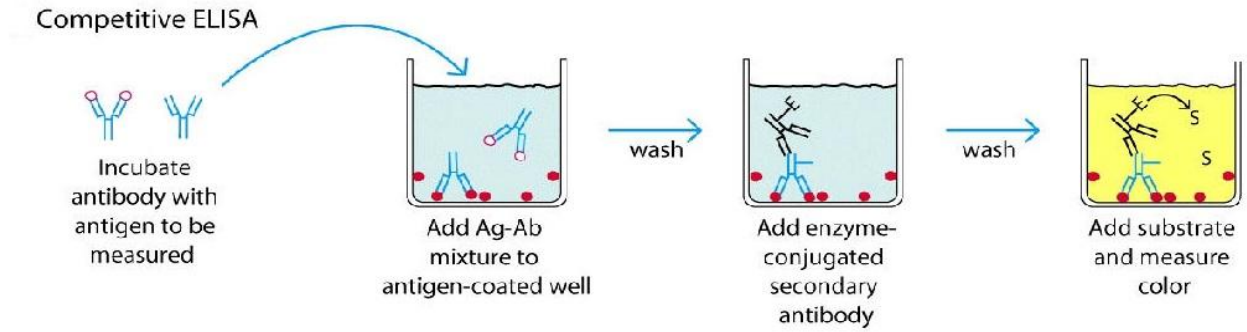
Sandwich ELISA

- Antigen can be measured by a sandwich ELISA.In this technique, the antibody (rather than the antigen) is immobilized on a microtiter well.
- A sample containing antigen is added and allowed to react with the immobilized antibody.After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen.
- After any free second antibody is removed by washing,substrate is added, and the colored reaction product is measured.



Competitive ELISA

- Antibody is first incubated in solution with a sample containing antigen.
- The antigen-antibody mixture is then added to an antigencoated microtiter well. The more antigen present in the sample,the less free antibody will be available to bind to the antigen-coated well.
- Addition of an enzyme-conjugated secondary antibody (Ab2) specific for the isotype of the primary antibody can be used to determine the amount of primary antibody bound to the well as in an indirect ELISA.

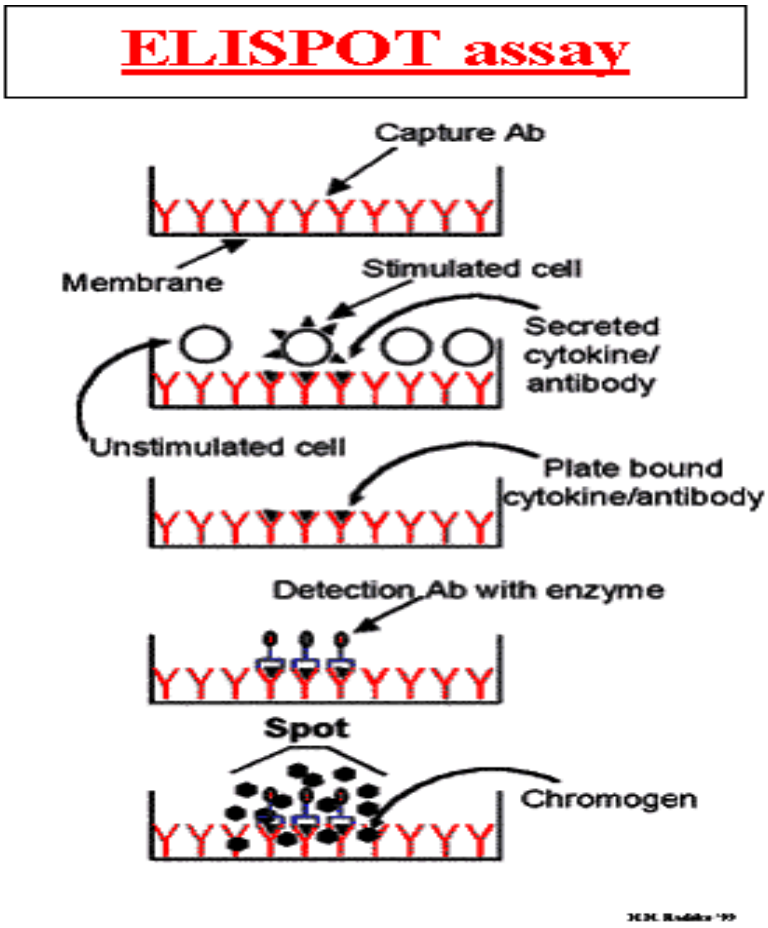


Application

- ELISA is a very sensitive laboratory method and can be quantitative when used in conjunction with standard curves.
- It can quantify the amount of antigen or antibody present in a given sample

ELISPOT ASSAY

- A modification of the ELISA technique is called the ELISPOT assay .
- This technique is used for quantitative determination of the number of cells in a population that are producing antibodies specific for a given antigen or an antigen for which one has a specific antibody
- A well is coated with antibody against the antigen of interest, a cytokine in this example, and then a suspension of a cell population thought to contain some members synthesizing and secreting the cytokine are layered onto the bottom of the well and incubated.
- Most of the cytokine molecules secreted by a particular cell react with nearby well-bound antibodies. After the incubation period, the well is washed and an enzyme-labeled anti-cytokine antibody is added.
- After washing away unbound antibody, a chromogenic substrate that forms an insoluble colored product is added.



- The colored product (purple) precipitates and forms a spot only on the areas of the well where cytokine-secreting cells had been deposited. By counting the number of colored spots, it is possible to determine how many cytokine-secreting cells were present in the added cell suspension.

Avidin-biotin immuno detection

- There are several ways in which the avidin- biotin interaction can be used as a detection system.
- The method of detection can be chosen based on its convenience or its detection sensitivity.
- The three basic designs are the labeled avidin-biotin (LAB), the hndged avidin-biotin (BRAB) system and the avidin-jbiotin complex (ABC) system.

- These methods can be used in ELISA, blotting, or immunohistochemical staining techniques.
