SECONDARY BINDING TESTS

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INTRODUCTION

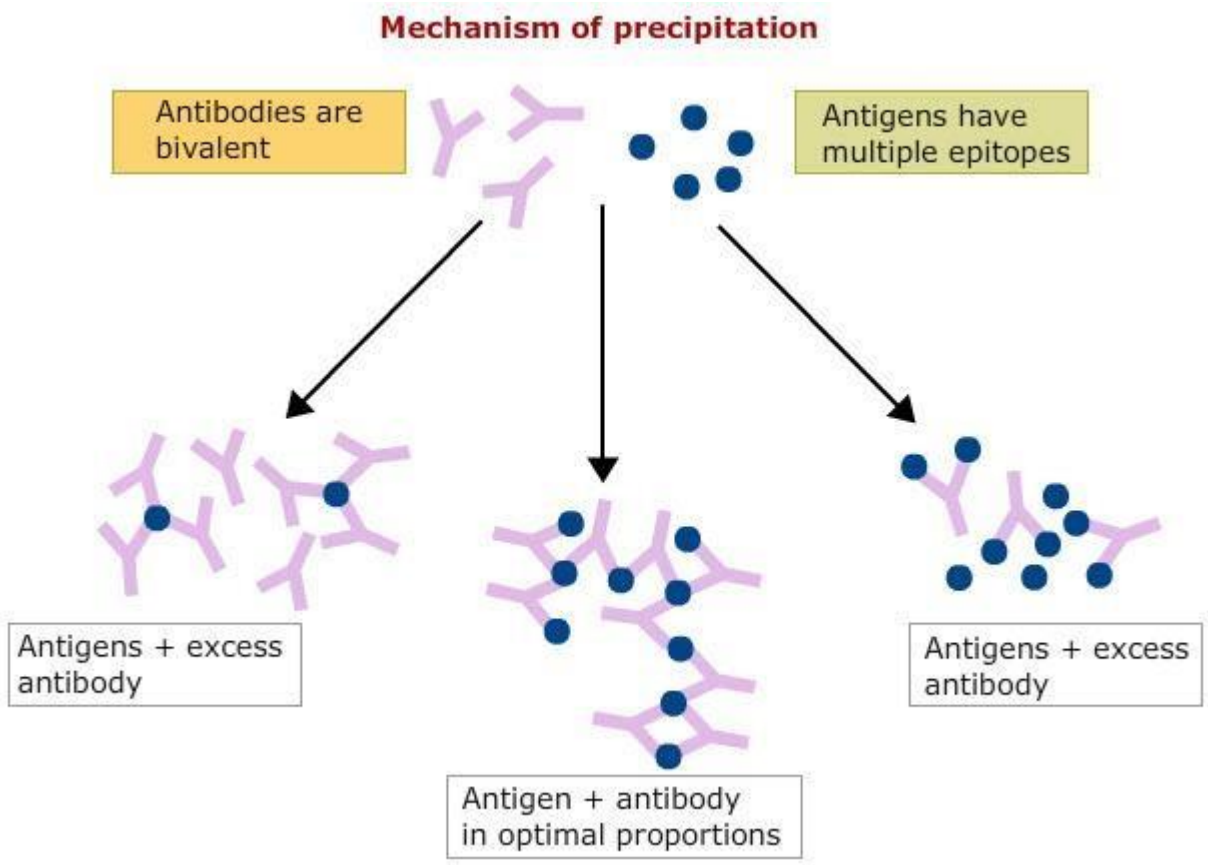
- Here the reaction between antigen and antibody is commonly followed by a second reaction.
- The antigen antibody complex formed a stable complex which can be visualized.
- Thus, if antibody combine with soluble antigens in solution, the resulting complexes may precipitate.
- If the antigens are particulate (eg: bacteria, RBCs), then antibodies will make them clump or agglutinate.
- If the antibody can activate the classical complement pathway and the antigen is on a cell surface, then lysis of the cell may result.

The secondary binding tests are

- Precipitation test
- Agglutination test

PRECIPITATION TEST

- If a solution of *soluble antigen* is mixed with a strong antiserum, the mixture becomes cloudy within a few minutes and then flocculent. Finally, a precipitate which consists of antigen – antibody complexes settles to the bottom of the tube.
- Antibodies that aggregate soluble antigens are called *precipitins*.
- For formation of an antigen – antibody lattice or complex (i) antibody must be bivalent (ii) antigen must be either bivalent or polyvalent i.e., it must have at least two copies of the same epitope or have different epitopes that react with different antibodies present in polyclonal antisera.
- If increasing amounts of soluble antigen are mixed with a constant amount of antibody, no obvious precipitate is formed at low antigen concentration. As the amount of antigen increases, larger quantities of precipitate form until the amount are maximal. With further addition of antigen, the amount of precipitate gradually diminishes until none is observed in tubes with excess antigen (Horse antibodies produce a distinct flocculation over a very narrow range of antigen concentration due to IgG (T) subclass).
- Where there is excess antibody, each antigen molecule is covered with antibody, preventing cross linkage and precipitation. When the reactants are in optimal proportion (*i.e.* in the equivalence zone), the ratio of antigen to antibody is such that cross linking and lattice formation is extensive. As this lattice grows it becomes insoluble and eventually precipitates. In antigen excess, each antibody binds two antigen molecules and further cross linking is impossible, and since these complexes are small and soluble, no precipitation occurs.



- The following are precipitation test
  - The precipitin ring test
  - Gel diffusion test/ Agar gel immunodiffusion tests [AGID]/ Agar gel precipitation tests [AGPT]
    - Single diffusion in one dimension (Oudin test)
    - Double diffusion in one dimension (Oakley – Fulthorpe test)
    - Single diffusion in two dimensions (Mancini test)
    - Double diffusion in two dimensions (Ouchterlony test)
  - Immunoelectrophoresis
    - Rocket electrophoresis
    - Counter immunoelectrophoresis (CIE)/Counter current electrophoresis/Cross over electrophoresis/Counter electrophoresis
    - Two dimensional Immunoelectrophoresis/2 way immunoelectrophoresis

THE PRECIPITIN RING TEST

- The antigen is carefully layered over the antiserum, without mixing so that an interphase is formed. Diffusion of each reagent occurs into the other.
- If the system is homologous, precipitation will occur at the point in the tube where the proper ratio of antigen to antibody is reached.
- **Example:** Ascoli's test for anthrax diagnosis.

GEL DIFFUSION TEST/AGAR GEL IMMUNODIFFUSION TESTS(AGID)/AGAR GEL PRECIPITATION TESTS [AGPT]

- A simple method of demonstrating precipitation of antigen by antibody is immunodiffusion or gel diffusion.
- Addition of agar to one or both reagents or between the reagents retards the rate of diffusion and permits formation of multiple distinct lines of precipitate if several antigenic substances and their antibodies are present.
- The forms of precipitation in gel commonly used are
    - Single diffusion in one dimension (Oudin test)
    - Double diffusion in one dimension (Oakley – Fulthorpe test)
    - Single diffusion in two dimension (Mancini test)
    - Double diffusion in two dimension (Ouchterlony test)

SINGLE DIFFUSION IN ONE DIMENSION(OUDIN TEST)

- The antiserum (antibody) is incorporated in melted agar and mixture is poured into a tube and allowed to solidify.
- Antigen solution is placed above the agar.
- The precipitin band appears in the agar.

DOUBLE DIFFUSION IN ONE DIMENSION(OAKLEY-FULTHORPE TEST)

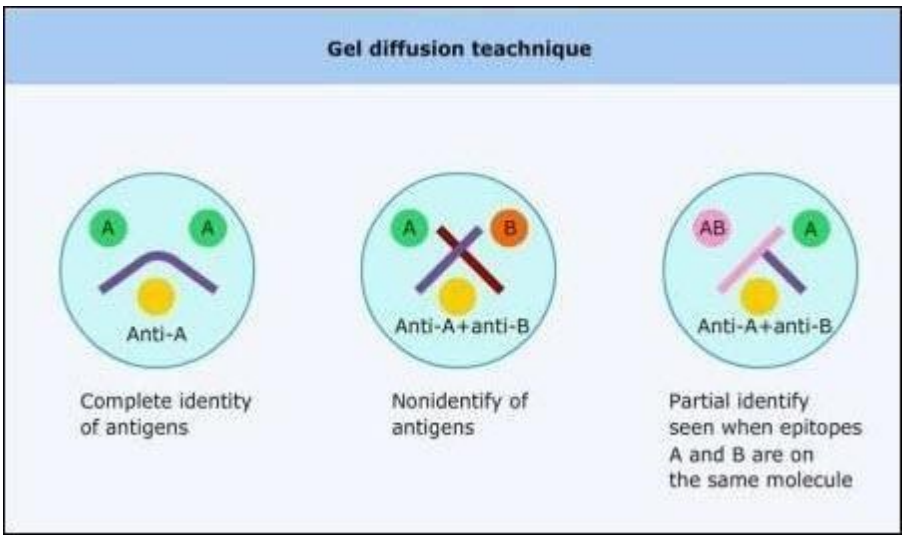
- Antibody (antiserum) is incorporated in agar, poured into a tube and allowed to harden.
- A second layer of agar without antibody is placed above and allowed to solidify.
- Antigen solution is placed above the agar.
- The precipitin band appears in the plain agar column.

SINGLE DIFFUSION IN TWO DIMENSION(MANCINI TEST)

- Antibody is incorporated into agar, poured into a glass plate to form a uniform layer.
- Circular wells are cut into the agar and antigen is introduced into the wells.
- Ring shaped bands of precipitates from concentrically around the well.
- This test is often used to quantitate the amount of antigen present and is read by measuring the size/area of the precipitin ring (figure).

DOUBLE DIFFUSION IN TWO DIMENSION (OUCHTERLONY TEST)

- In this test two or more wells are cut into the agar.
- Antibody is added to one well and antigen to the other wells.
- Lines of precipitation form where antigen and antibody which are both diffusing into the agar meet in optimal proportions.
- This test can be used to find out relationship between antigens.
- The possible outcomes of the test are
    - The two precipitin lines join - line of identity - the two antigen are identical
    - The two lines cross each other - line of non identity - the two antigens are completely different
    - The lines merge with a *spur* formation - a partial identity exists indicating that the antigens share an epitope but one of the antigens possess an epitope not present in the other .
- *Coggins test* is a gel diffusion test used to diagnose equine infectious anemia (EIA) in horses.



IMMUNOELECTROPHORESIS

- Involves the electrophoresis of the antigen mixture in agar gel in one direction.
- A trough is then cut in the agar gel to one side and parallel to the line of separated protein.
- Antiserum is placed in this trough and allowed to diffuse laterally.
- When the diffusing antibody encounters antigen, curved lines of precipitates are formed.
- One arc of precipitate forms for each constituent in the antigen mixture.
- Immunoelectrophoresis can resolve normal serum proteins into 25-40 distinct bands and can be used to identify absence of normal serum proteins and also excess of some proteins (*Example:* in myeloma)

ROCKET ELECTROPHORESIS

- Instead of allowing the antigen in wells to diffuse into the antiserum containing agar (Mancini test), if it is driven into the antiserum agar by electrophoresis, the ring of precipitate around each well becomes deformed into a rocket shape and length of the rocket is proportional to the amount of antigen placed in the well. This technique is called rocket electrophoresis.

COUNTER IMMUNO ELECTROPHORESIS (CIE)/COUNTERCURRENT ELECTROPHORESIS/CROSS OVER ELECTROPHORESIS/COUNTER ELECTROPHORESIS

- Performed in agar gels.
- Gammaglobulins are exceptional in their cathodic migration. Most of the other proteins move to anode.
- By applying a voltage across the gel the antigen and antibody move towards each other and precipitate.
- The principle is same as in double diffusion, but is much faster and sensitivity is more.
- It can be used for the rapid diagnosis of many diseases.

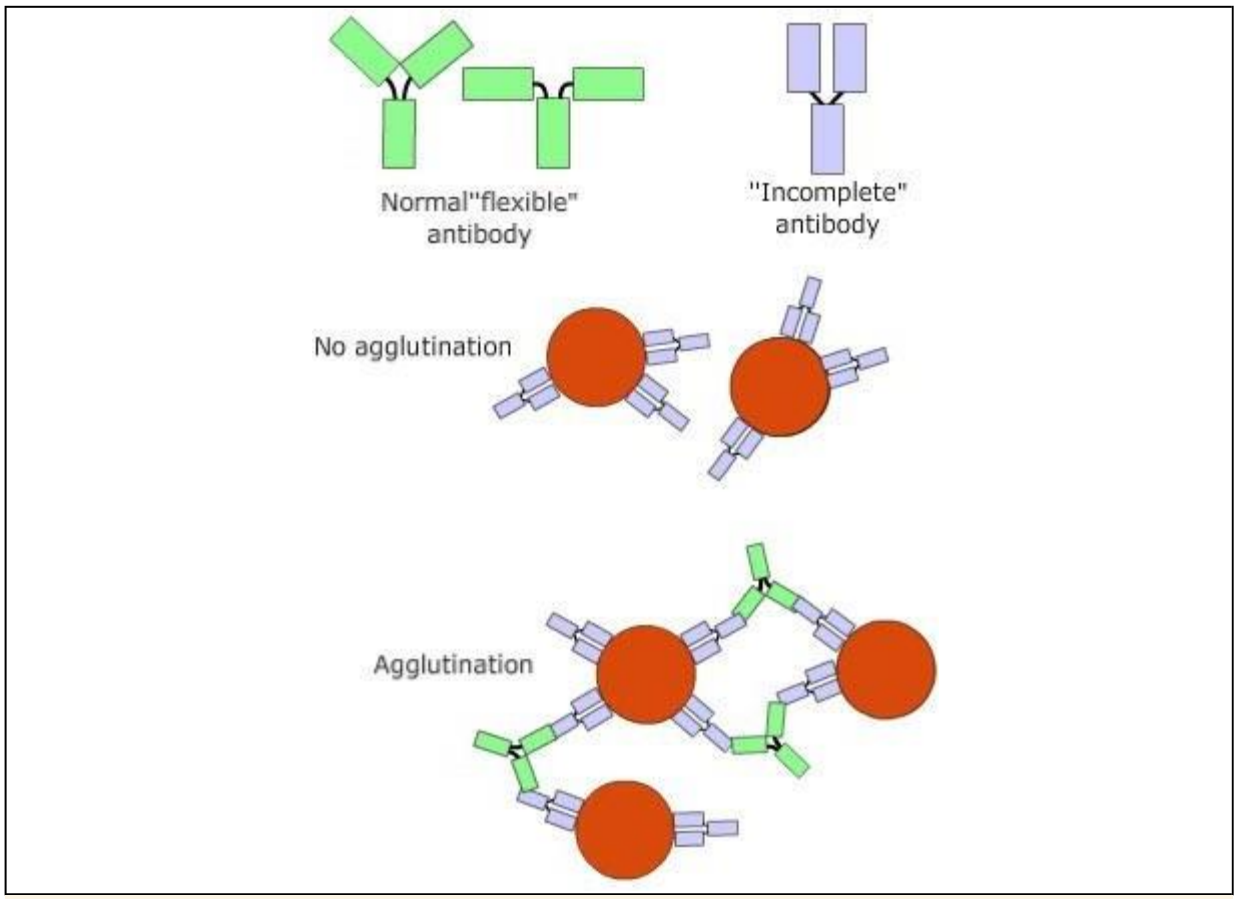
TWO DIMENSIONAL IMMUNOELECTROPHORESIS/TWO WAY IMMUNOELECTROPHORESIS

- In this method a strip of agarose containing electrophoretically resolved proteins is placed along one edge of the plate.

- Molten agarose containing antiserum is poured over the rest of the plate.
- When the plate has gelled, the antigens are forced into the antibody layer by electrophoresis.
- Migration is continued until each antigen is precipitated by its specific antibody .

INTRODUCTION- AGGLUTINATION TESTS

- The interaction between antibody and a *particulate antigen* results in visible clumping called agglutination.
- Antibodies that produce such reactions are called *agglutinins*. IgM is more efficient than IgG in causing agglutination.
- Agglutination reactions are similar in principle to precipitation reactions; they depend on the cross linking of polyvalent antigens.
- Antigen and antibody must be present in correct proportion (Zone of equivalence) for agglutination to occur.
- Just as an excess of antibody inhibits precipitation reactions, such excess can also inhibit agglutination reactions. This inhibition is called the *prozone effect*.
- The causes of prozone effect are
    - In antibody excess, each antigenic particle may be so coated by antibody that agglutination is inhibited.
    - Presence of non agglutinating antibodies called *incomplete antibodies*.
- The lack of agglutinating activity of such antibodies may be because of the restricted flexibility of the hinge region and or the epitopes that they bind lie deep with in the antigen.
- For detecting presence of non agglutinating antibodies on the surface of particles a direct antiglobulin test may be used.
- The washed particles are mixed with antiglobulin and if immunoglobulins are present, agglutination will occur.
- Post zone effect is seen when antigens are present in excess.
- Agglutination does not occur in prozone and post zone.



PREREQUISITES FOR AGGLUTINATION

- Particles used as antigens should have size of > 200 – 250 nm and should remain in suspension for a reasonably long time (48 hours minimum).
- Antibody should be directed to the target surface antigen
- Antigen and antibody should be present in optimal proportions
- An electrolyte or buffer is necessary for the reaction
- Agglutination of bacteria as a test to detect presence of bacterial antibody in sera is an important application of the agglutination reaction.
- Many bacteria such as Brucella, Salmonella, Escherichia, Proteus etc. form smooth suspensions in buffered saline.
- When incubated with antibody against surface antigen like those of flagella, capsular material or cell wall components, the bacteria agglutinate to form clumps.
- Agglutination test is also used for identification of bacterial strains using specific antisera.
- Agglutination can be done in slides or in tubes and hence there is the slide agglutination test and tube agglutination test.

PASSIVE HAEMAGGLUTINATION

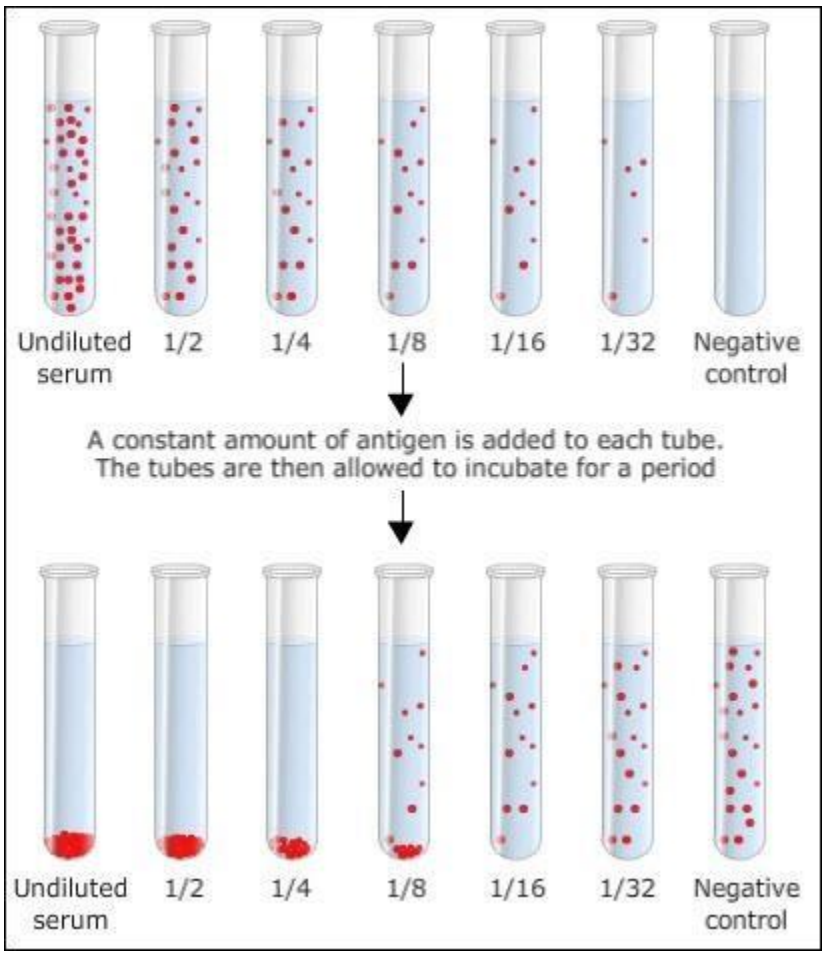
- Since agglutination is a much more sensitive technique than precipitation, it is sometimes useful to convert a precipitating system to an agglutinating one.
- In passive haemagglutination, antigen coated red blood cells are prepared by mixing a soluble antigen with RBCs that have been treated with tannic acid or chromium chloride, both of which promote adsorption of antigen to the surface of the cells.
- The antigen coated RBCs are agglutinated by specific antibody to the adsorbed soluble antigen.
- In the actual test, serum containing antibody is serially diluted into microtitre plate wells and the antigen coated RBCs are than added to each well.
- Wells showing a layer of uniformly agglutinated cells on the bottom of the well are positive.
- In negative reaction, compact sharply demarcated discs of sedimented cells are seen at the bottom of the well.
- Passive haemagglutination is used for assessing the antibody titre in vaccination against Pasteurellosis.

LATEX AGGLUTINATION

- Latex beads are coated with either antigen or antibody.
- Sensitized latex beads are added to the clinical samples.
- In positive cases, antigen and antibody combines resulting in clumping of latex beads leading to agglutination.
- This test can be read by eye with in a minute or so.

VIRAL HAEMAGGLUTINATION AND HAEMAGGLUTINATIONINHIBITION

- Some viruses can bind and agglutinate mammalian and avian species of RBCs. This assists in characterization of unknown viruses.
- *Specific antibodies* against the virus inhibit haemagglutination. This is called haemagglutination inhibition (HI). Hence HI can be used for accurate identification of different haemagglutinating viruses and also to determine levels of antibodies to haemagglutinating viruses. Examples of haemagglutinating viruses are Newcastle Disease virus, Egg Drop Syndrome virus, canine parvo virus, rabies virus etc.
- *Bifunctional antibodies:* It is made by breaking the bonds between the two heavy chains so that two identical halves are formed. Then two halves from different immunoglobulins are joined to produce a molecule that can cross link two different epitopes. Eg: when a bifunctional antibody, one half of which is directed against dog RBCs and other half against adult heart worm (*Dirofilaria immitis*) is mixed with whole blood from a heartworm infected dog, it cross links the heartworm antigen to RBCs resulting in visible haemagglutination in a few minutes.



COMPLEMENT FIXATION TEST

- The activation of the complement system by antiserum bound to antigen results in the generation of membrane attack complexes that can disrupt cell membranes.If the antibody is bound to RBC, then hemolysis occurs. This phenomenon can be used to measure serum antibody levels in a test called the complement fixation test.
- First the antigen and serum under the test (heated to 56°C for 30 minutes to inactivate complement) are incubated in the presence of normal guinea pig serum (it acts as the source of complement).
- After the antigen - antibody - complement mixture reacts, the amount of free complement is measured by adding an indicator system consisting of antibody coated sheep RBCs.
- Lysis of these cells (seen as a transparent red solution) is a negative result since it indicates that complement was not activated and that antibody was absent from the test serum.
- Absence of lysis indicated by a cloudy RBC suspension or button formation indicates that complement was consumed or fixed. This is a positive test.
- This test is applied for the diagnosis of many viral and bacterial diseases. Ex. FMD in animals, Japanese encephalitis in man etc.

INTRODUCTION- ASSAYS IN LIVING SYSTEMS

- If an organism or antigen possesses biological activity, antibody can be measured by their ability to neutralize this activity.
- The activities that may be neutralized include hemolysis of RBCs, lysis of nucleated cells, and disease or death in animals.
- Reactions such as these are subject to high degree of variability.

NEUTRALIZATION TEST

- Neutralization tests estimates the ability of antibodies to neutralize the biological activity of antigen when mixed with it *invitro*.
- These tests may be used to identify bacterial toxins, such as toxin of *Clostridium perfringens* or *Staphylococcus Sp*.
- Viruses may be prevented from infecting cells after specific antibodies has combined and blocked (neutralized) theircritical attachment sites.
- Neutralization tests are highly specific and extremely sensitive.

PROTECTION TEST

- Form of neutralization test done *in vivo*.
- The protective properties of a specific antiserum are measured by administering it in increasing dilutions to a group of test animals, which may then be challenged with a standard dose of pathogenic organism or toxin.
- These tests should be done in large number of animals to get meaningful results.