LABORATORY METHODS

DETECTION OF ANTIGEN (ag) – ANTIBODY (ab) INTERACTIONs

SEROLOGIC REACTIONS IN VITRO (ANTIGEN-ANTIBODY) REACTIONS PROVIDE METHODS FOR THE DIAGNOSIS OF DISESE AND FOR THE IDENTIFICATION & QUANTITATION OF ANTIGENS & ANTIBODIES

ONE OF THESE SEROLOGIC REACTIONS IS CALLED **:**

PRECIPITATION REACTIONs

DEFINITIONs

Precipitation : In a solution, it means; that, "soluble" reactants (ag-ab) should be aggregated, condensed, and fall, thus; separated from a solution.

Precipitin : An antibody (soluble) that interacts with an antigen (soluble) to cause precipitate.

Precipitinogen : An antigen (soluble) that induces the formation of a specific precipitin (soluble antibody).

Lattice : A three-dimensional grid (network) .

If the reactants (ags and abs) both are soluble, then how the reaction can precip]itate and can be seen (detected) ?.

Precipitation will develop, when the antigens (the antigens must have at least two epitopes per molecule) are crosslinked and forms a lattice. For the lattice to be formed, the bivalent antibody will bind to epitopes on two different antigens. A second ab molecule combine with the second epitope on one of the antigen molecules and a third epitope on another antigen molecule, so that the complex is formed.

When repeated so many times, the complex continues to grow "until" it is sufficiently large to become insoluble and precipitate. Because the antigen is soluble, a large "number" of molecules are required for lattice formation.

Is there any effect on the precipitation by changing the amount of antigens (concentrations) ? .

When the ag concentration is very low and that of the ab is relatively superabundant (zone of ab excess), formation of "small" complexes occurs. If the mixture [reactants (ag-ab)] are centrifuged, residual abs will remain in the supernatant. This area (supernatant) containing excess antibodies is called PROZONE (Figure: 1-2).

A more antigen is added, large aggregates form, when there is neither antigen nor antibody in the supernatant, the situation is called EQUIVALENCE ZONE . This where the maximal precipitation occurs (Figure: 1-2).

With increasing the amounts of ag , the lattice size becomes too small to precipitate. This situation is called the POSTZONE (zone of ag excess). Instead of reaching the plateau, the curve comes back down to zero (Figure: 1-2).

FIGURE: 1.

Effect of increasing amount of ags with fixed amount of abs. The precipitation curve shows the maximal amount of precipitation in the zone of equivalence. This reaction is performed by using several tubes, each with the same concentration of ab. The tubes have an increased amount of ags added.

Figure: 2.

The formed lattice determines the amount of the precipitation in each zone of the precipitation curve. No or little precipitate is formed in the prozone (ab excess prevents crosslinking of antige molecules. At the zone of equivalence, precipitate is formed because the lattice is large and insoluble. In the post zone, little or no precipitate is formed because lattice formation does not occur in antigen excess.

General information about the precipitation reactions.

Precipitation reaction can occurs using polyclonal abs or mixture of monoclonal antibodies. If the antigen is monovalent or a single monoclonal ab is used, no lattice will form. Precipitation reactions may require hours or days to become visible, depending on the type of precipitation reaction.

In general, precipitation techniques are not as sensitive as other techniques because a "sufficient number" of antigen and antibody molecules "must" be "crosslinked" in order to see the precipitate.

TYPES OF PRECIPITATION REACTIONS

There are several precipitation test systems. All are used in the clinical laboratory. Each system can detect antigens or antibodies, depending on the configuration of the assay. Moreover, each system has different sources of error that must be taken into account when performing a particular laboratory procedure. In all optimal lattice forms in the zone of equivalence. These techniques are :

1st. PASSIVE DIFFUSION :Effective automatically without the need for voluntary action.

One. Fluid

1. Double diffusion
2. Capillary tube precipitation

Two. Gel

1. Double diffusion (Ouchterlony).
2. Single-diffusion radial immunoassay (RID).

2nd. ELECTROPHORESIS : Voluntary action is the electricity.

1. Countercurrent immunoelectrophoresis (CIEP).
2. Immunoelectrophoresis (IEP).
3. Immunofixation electrophoresis (IFE).
4. Rocket technique (Laurell).

Fluid-Phase Precipitation

The "passive diffusion" of "fluid-phase" (double diffusion) is one of the first precipitation reactions antigen and antibody. This double diffusion method in a capillary tube layers and antigen solution over an antibody solution. Both ag and ab will diffuse towards each other; at the interface, when ab recognize ag, precipitate forms. The amount of the precipitate is proportional to the concentration of both the ag and ab (Figure 3).

This procedure is used to identify unknown ag or unknown ab. If an ag is to be detected, a fixed amount of known reagent "ab" is placed in the capillary tube; the greater the amount of precipitate formed, the greater the concentration of ag. If a double diffusion method is used to detect ab, a fixed amount of known reagent ag is used.

FIGURE 3.

The ag and ab solutions are placed on top of each other in a capillary tube. After diffusion the ag and ab precipitate in the zone of equivalence. In sample A and B, some precipitate is formed at the interface, with B having more than A. In sample C there is no precipitate.

Precipitation Reactions in Gel

The "passive diffusion" of the "gel phase" (gel is used as a semi-solid medium, it is a gelatinous colloid in which a solid is dispersed in a liquid. Typically less than 1% of the total is the solid, and heat is required to dissolve the solid and to trap the liquid, creating the semisolid). The gel contains "pores" that allow the movement of molecules. In immunoprecipitation reactions, the gel is a derivative of agar and is called agarose.

Agar is a complex sulfated polysaccharide derived from algae. Agar can be purified into agaropectin, containing carboxylic acid and acid sulfate side chains, and agarose, containing few ionizable groups.

Agarose is preferred in immunologic reactions because its neutral nature does not interface with the ag or ab reactants, and it has low endosmosis. Agarose gel allows soluble ag and /or ab to diffuse through the pores until the ag and ab reach the optimal concentration for lattice formation.

The molecular size determines the rate of diffusion through the gel. In general, smaller molecules move through the gel faster than larger molecules. A mixture of ag and /or abs may result in several precipitin lines; each ag and the corresponding ab will form a lattice in its zone of equivalence. The diffusion rate also depends on temperature, gel viscosity, and hydration, electroendosmotic effect, and the interactions between the gel matrix and reactants.

Double diffusion in Gel

The Ouchterlony Technique : Both the ag and ab diffuse in a gel.

1. **Agarose gel is placed on a solid surface (petri dish, glass slide, or plastic plate) and allowed to solidify.**
2. **Wells are cut into the gel and the agarose plug is removed.**

{ Typically a central well is surrounded by multiple wells}

*** If ag is to be detected, a known reagent ab is placed in the center well and the unknown samples are placed in the surrounding wells.**
*** If ab is to be detected, unknown ag is placed in the center.**

3. **After each of the samples and reagent have been added to the appropriate wells.**

{Diffusion occurs, and a line of precipitation forms at the zone of equivalence}

*** If multiple wells of ag are positioned around an ab well on the same plate, several patterns of reactivity may be observed (Figure 4).**

FIGURE 4

(A) Ag a and b are identical. Where the lines of precipitate (solid, continuous, smooth line) come together on the plate, a smooth curve is formed (Identity). (B) Ags a and b are not identical. In the area on the plate where the two ags may react with ab, the lines of precipitate will cross through each other (Non-Identity) . (C) Ags a and b are similar but not completely identical. Where the lines of precipitate join, the line is not completely smooth. The spur points to the simpler ag (Partial Identity).

Applications

This technique has been used in the clinical laboratory to detect abs to specific nuclear components in autoimmune diseases such as systemic lupus erythematosus (SLE). This can be used to qualitatively detect the presence of the ab, and serial dilutions can react to establish a titer.

Sources of errors

Irregular patterns caused by the overfilling of wells, irregular well punchig, and nonlevel incubation. Other problems may include gel drying, so that the gel is not as porous, and increased room temperature, which causes greater diffusion. Ag or Ab degradation caused by bacteria or fungal contamination will result in diminished precipitation. Ag or Ab excess may yield false negative results. This may be overcome by using several conce. Of both ag & ab, so that the combination will be in the zone of equivalence.

RADIAL IMMUNODIFFUSION (RID)

RID a commonly used gel precipitation technique (Figure 5). In this technique :

1. **Antiserum is added to the liquified gel, which is poured into a plate and allowed to solidify by cooling to room temperature.**

   {The antiserum should be monospecific, have high affinity and avidity, and excellent precipitating ability; generally IgG abs are best}

2. **The antigen is added to wells cut into the agar.**

   {The antigen diffuses in all directions from the well, and the precipitate is a concentric ring. The incubation period for the diffusion depends on the molecular weight of the antigen; larger molecules diffuse more slowly, requiring more time for full diffusion and maximum precipitin ring formation}

FIGURE 5

(1st) The antigen is placed in the well; it diffuses into the agarose in all directions. (B) The area around the well where precipitation occurs is the area of the zone of equivalence between the antigen and antibody. The diameter of the area of precipitation (including the well diameter) is measured to determine the concentration of antigen.

Radial immunodiffusion (RID) can be constructed with one of two incubation times-the kinetic diffusion, or Fahey, method and the endpoint diffusion, or Mancini, method.

{Regardless of which method is performed, three standards are used-generally a high concentration, a normal concentration, and a low concentration}

In the kinetic diffusion method:

- **The diameter of the precipitin rings is measured at 18 hrs.**
- **The logarithm of the concentration of the standards is proportional to the diameter of the precipitin ring.**
- **Using semilogarithmic paper, the *y* axis is the analyte concentration and the *x* axis is the diameter of the ring (including the well diameter).**

The standard values are plotted and a line is drawn point to point. The analyte concentration of the patient and the control sera may be read from the graph. Figure 6 is an example of a kinetic diffusion graph.

FIGURE 6

In the kinetic radial immunodiffusion method, the diameter of the ring is plotted versus the concentration on semilogarithmic graph paper. Values of high, normal, and low reference sample are plotted, and the points are connected. Concentrations of unknown samples may be determined using this graph. For example, if the patient sample has a diameter of 5mm, the sample contains 550 mg/dL of IgG. If the patient sample has a diameter of 8mm, the sample contains 1800 mg/dL.

In the endpoint method :

- **The antigen is allowed to diffuse fully to achieve maximal precipitation.**
- **The time needed varies, depending on the molecular weight of the protein being measured. For example, IgG quantitation requires a 48-hrs incubation, whereas IgM requires a 72-hrs incubation.**
- **Using linear graph paper, the concentration of antigen is plotted on the *y* axis and the diameter squared of the precipitin ring is plotted on the x axis. The points are connected by the line of best fit (Figure 7). The concentration of the unknown sera is read from this graph.**

FIGURE 7

In the endpoint method, the square of the diameter of the ring is plotted against the concentration of antigen on linear graph paper. Values of high, normal, and low reference samples are plotted and the line of best fit is drawn. Concentrations of unknown samples may be determined using this graph. For example, if the patient sample has a diameter of 5 mm, the sample contains 585 mg/dL of IgG. If the patient sample has a diameter of 8 mm, the sample contains 1520 mg/dL of IgG.

Patient values can be obtained only if the precipitin ring of the patient sample is within the range of measured rings for the standard sera. Only valid readings are obtained between the highest and lowest standards.

*** If the patient results are outside of these limits (highest & lowest standard sera) , the assay must be repeated.**

*** If the diameter of the precipitin ring of the patient sample is greater than that of the highest reference serum, the line should not be extended to obtain the patient concentration, since linearity above the reference line cannot be guaranteed. It is recommended that the serum be diluted with normal saline and the assay repeated. The result obtained should be multiplied by the dilution factor.**

*** If the diameter of the precipitin ring of the patient sample is below the lowest reference standard, the results should be reported as less than the standard value or the sample should be assayed on a low-plate. A low-level plate contains less antibody in the gel so that lower concentrations of antigen are detected.**

Sources of errors

These include:

1. **Overfilling or underfilling the wells.**
2. **Spilling the serum on the gel.**
3. **Nicking the side of the well when filling.**
4. **Improper incubation time and temperature.**

All of these will lead to inaccurate quantitation, requiring the sample to be reassayed (Figure 8).

FIGURE 8

Ring patterns on a RID plate should be equal around the well. In this figure, wells 1 through 4 are normal. Well 5 has more precipitate on one side, which indicates that some of the sample

spilled on the gel. There is no precipitate around well 6, which indicates that the well was not filled, was unfilled, or that the sample contained little or no analyte. Well 7 has an irregular shape on one side, which indicates that the well was nicked during filling. Well 8 is an of double precipitin rings.

COUNTERCURRENT IMMUNOELECTROPHORESIS (CIEP)

A third precipitation reaction in gel is countercurrent immunoelectrophoresis (CIEP).

1. **Gel is poured onto a plate and cooled.**
2. **Two columns of wells are cut and evacuated; antigen is placed in one well and the antibody is placed in the other well.**
3. **The plate is placed in an electric field, causing migration of the antigen and antibody based on charge.**
4. **At pH 8.6 the antigen will migrate toward the anode (+ ve) and the antibody toward the cathode (-ve).**

{At equivalence, precipitation occurs (Figure 9).

FIGURE 9

In countercurrent electrophoresis, antigen and antibody wells are placed opposite to each other. In this figure, unknown antigen is detected using known reagent antibody. The plate is electrophoresed and a precipitin line is formed at the zone of equivalence, which may not always be midway between the two wells. Samples 1, 3 and 5 have antigen present and in equal concentration to the known antibody. Sample 2 has antigen present, but at a lower concentration

than the antibody. Sample 4 has no antigen present. Sample 6 has antigen present at higher concentration than the antibody.

{The electric field increases the rate of migration of the antigen and antibody, thereby accelerating the visibility of the precipitate}

This qualitative procedure is used to detect autoantibodies, antibodies to infectious agents, and certain microbial antigens. This method can be semiquantitative by using serial dilutions.

*** If the antigen is diluted and the concentration of antibody is constant, the precipitin line moves closer to the antibody well as the concentration of the antigen increases. The antibody may be diluted instead of the antigen, with the line of precipitation moving toward the antigen well.**

Sources of errors

These sources of errors are related either to electrophoresis of precipitation.

Electrophoresis errors are:

1. **The reversal of the wells so that the current is applied in the wrong direction. The antigen and antibody will migrate to the edge of the plate rather than the center.**
2. **Improper pH of the buffer, which may alter the net charge of the antigens and antibodies, thus affecting migration, and insufficient electrophoresis time, which will not allow complete migration to occur.**

Errors related to precipitation are :

1. **There will be no or reduced precipitate in the prozone or postzone.**

2. **When the plate is prepared, it is important that the two lines of wells are parallel so that the antigen and antibody migration paths meet. If these two lines were not prepared well, the ag and ab might not meet at all.**

IMMUNOELECTROPHORESIS (IEP)

Immunoelectrophoresis is a gel electrophoretic technique commonly used in the clinical immunology laboratory. This procedure uses both electrophoresis and double diffusion (Figure 10).

Patient serum is placed in a well and electrophoresed. The parameters of IEP are the same as serum protein electrophoresis so that the separation is the same. Albumin migrates toward the anode (+ve) and the immunoglobulins migrates to the $\alpha 2$, β, and γ globulin regions. Anti-human serum is placed in the trough, and the antiserum and the separated patient proteins diffuse toward each other.

Precipitin arcs form at the zone of equivalence between the antigen and specific antisera. Anti-total human serum is a mixture of antibodies against all serum proteins and produces many precipitin arcs.

If a nonspecific antiserum is placed in the trough, then only one arc will be formed if the particular serum component is present. The plate may be stained and photographed. The precipitation patterns of identity, non-identity, and partial identity are observed. A normal control serum is performed simultaneously, so that the two may be compared.

FIGURE 10

The first step required to form a precipitin arc in immunoelectophoresis is to separate proteins in patient and control sera. The proteins migrate to the same area as in routine protein electrophoresis. The second step requires total anti-human serum, which contains specific antibodies to different serum proteins to be added to the trough and allowed to diffuse through the gel. Wherever the patient or control sera and each specific antibody in the total anti-human

serum are in the zone of equivalence, a precipitin arc is formed. The relative serum protein concentration determines the size of the arc. For example, the anti-albumin in the trough diffuse toward the albumin of the patient control serum. Where the two meet, the precipitin arc forms. Because more albumin is present than IgG in normal serum, the size of the arc for albumin is larger than that for IgG.

This procedure (IEP) is relatively insensitive to the antigen/antibody ratio, so it has been used to detect free light chains in antigen excess. It can also be used to screen for abnormalities in immunoglobulin classes (Figure 11). Because the size of the arc indicates the amount of immunoglobulin present, the procedure is semiquantitive. The shape and position of precipitin arcs provide clues as to the monoclonalality of a protein.

FIGURE 11

In some cases more information may be obtained from serum immunoelectophoresis by using monospecific antibodies. In each case, the sample is placed in the well and electrophoresed; monospecific antiserum is placed in the trough. (A) An example of normal patient serum

compared with the control serum. The two patterns are identical. Because the κ and λ antisera will be in all regions where IgG, IgM, IgA, and IgD are found. (B) An example of a patient with the IgM κ paraprotein. Note the increase in the precipitin arc of the patient serum with anti-μ and anti-κ anti-serum when compared with the precipitin arcs of the control.

IEP may also be used to identify urine proteins. Urine is placed in the well and electrophoresed, troughs are filled with anti-sera, and diffusion occurs. Free light chains and intact immunoglobulin molecules can be characterized (Figure 12).

FIGURE 12
Urine immunoelectrophoresis from the same two patients whose sera were evaluated in Figure 11 are shown here. (A) Normally urine contains little protein; therefore, no precipitin arcs are formed. The control used in this procedure is normal human serum. (B) The patient urine has increased μ and κ precipitin arcs compared with the control. This is consistent with the serum findings in figure 11. No other arcs compared because no other urine proteins are present in detectable quantities.

Sources of errors

The sources of errors associated with this procedure (IEP) are :

1. **Prolonged diffusion, which results in artifacts, especially at the anode (+ve) & cathode (-ve).**
2. **Excess antibody may result in multiple concentric arcs that could be mistaken for multiple antigen reactions.**

IMMUNOFIXATION ELECTROPHORESIS (IFE)

Immunofixation electrophoresis (IFE) is one of the useful gel electrophoretic technique useful in the clinical laboratory.

1. **Serum, urine, cerebrospinal fluid (CSF) are electrophoresed.**
2. **Antisera is applied.**
3. **A cellulose acetate strip impregnated with the antiserum is placed on the separated proteins (Figure 13).**

{The antiserum is diffuses into the gel rapidly, resulting in the precipitation of antigen-antibody complexes}

The resolution of immunofixation electrophoresis (IFE) is greater than that of immunoelectrophoresis (IEP). Compared with IEP, the <u>IFE technique is more sensitive to the antigen/antibody "ratio</u>".

In IFE serum dilution or antiserum dilution is necessary to produce the precipitin reaction. The dilution is necessary to produce the precipitin reaction. The dilution depends on the size of the monoclonal band in high resolution protein electrophoresis.

Urine and CSF must be concentrated to be in the zone of equivalence; typically urine is concentrated 25 times and CSF is concentrated 50 to 100 times. If there are air bubbles when the cellulose acetate is applied to the gel, diffusion can not occur at these points and a precipitation reaction may be missed.

FIGURE 13

(1st) After gel electrophoresis on the serum, urine, or cerebrospinal fluid. Cellulose acetate strip impregnated with antiserum are placed on the gel. There should be no bubbles between the strip and the gel so that the antiserum can completely diffuse into the gel. (B) After incubation to allow for diffusion, the cellulose acetate strip are removed and the precipitin bands are stained. As with other electrophoresis procedures, the stained area identifies the location of the specific protein as it would be found on routine protein electrophoresis. Because the κ and λ light chains are usually associated with all heavy-chain classes, the anti-

κ and anti-λ reactions will occur in all regions where immunoglobulin has been electrophoresed.

ROCKET TECHNIQUE

Another electrophoretic precipitation technique, used primarily in research and coagulation laboratories, is the rocket, or Laurell technique. This technique is used to quantitate antigens other than immunoglobulins. Antiserum is incorporated into the gel. The unknwon antigen is placed in the well and electrophoresed. As the antigen migrates through the gel, it combines with antibody. Precipitation occurs along the lateral boundaries and resembles a rocket (Figure 14). The total distance of antigen migration and precipitation is directly proportional to the antigen concentration.

FIGURE 14

In the Laurell rocket technique, the gel contains a specific antiserum. The samples are electrophoresed. The area where the antigen and antibody are in the zone of equivalence will

show precipitation. The rocket area may be quantitated. Sample 2 has the highest concentration of antigen; sample 6 has the least amount.

As previously discussed, a variety of procedures may be used in the clinical and research laboratories using precipitation techniques. The specific methodology used depends on the concentration of the antigen and whether quantitation is necessary.

Table 1 summarizes some of these tests and which procedure may be used for each.

Table 2 summarizes the advantages and disadvantages of each procedure.

TEST	METHOD
Antibodies to nuclear antigens	Double diffusion
Immunoglobulin quantitation	RID
IgG, total and subclasses IgA IgM IgD	
Complement proteins	RID
C3 C4	
Microbial antigens	CIEP
Monoclonal proteins (serum, urine, and cerebrospinal fluid)	IEP and IFE
Coagulation factors	Rocket technique

TABLE 1. Test Procedures Using Precipitation Techniques

RID, radial immunodiffusion; CIEP, countercurrent immunoelectrophoresis; IEP, immunoelectrophoresis; IFE, immunofixation electrophoresis.

TECHNIQUE	ADVANTAGES	DISADVANTAGES
Capillary tube precipitation	Easy to set up	Insensitive Reaction time long Semiquantitative
Radial immunodiffusion (RID) Quantitative Can detect only one antigen/plate	Sensitive (Kinetic: 18 hrs; endpoint:48 hrs)	Reaction time long
Double diffusion (Ouchterlony)	Can detect similarities among antigens	Semiquantitative Reaction time long
Countercurrent immunoelectrophoresis (CIEP)	More rapid than other tests	Semiquantitative
Immunoelectorphoresis (IEP)	Sensitive Less problem with ag/ab ratio	Semiquantitative
Immunofixation electrophoresis (IFE)	Sensitive Can detect genetic variations ag/ab ratio important among antigens	Semiquantitative
Rocket technique (Laurell)	Rapid reaction time Quantitative	Can detect only ag/plate

Table 2. **Advantages and Disadvantages of Precipitation Techniques**

APPLICATIONS

Radial Immunodiffusion

RID procedures may be used for :

1. **Quantitation of immunoglobulins, including subclasses of IgG.**
2. **Quantitation of complement components.**
3. **Quantitation of other serum proteins.**

Small laboratories use this quantitative method because, no capital equipment is needed. The only problem is the time required for the assay and the additional time needed if the results are out side of the standard limits. Currently, the majority of IgG subclasses are performed using RID.

Larger institutions, RID has been replaced by nephlometry. Immunoglobulin and complement proteins concentrations may be obtained in minutes rather than the hours or days required for RID.

BOTH METHODS (RIDs & NEPHLOMETRY) ARE ACCURATE AND REPRODUCIBLE

Immunoelectrophoresis

IEP procedures may be used to:

Qualitatively identify monoclonal proteins, including free κ and λ chains.

In this procedure, the antigen and antibody ratios are not critical. In addition to free κ and λ chains, all immunoglobulin classes may be detected in both serum and urine.

This test is especially useful to :

Evaluate hypergammaglobulinemia, such as in multiple myeloma.

Some indications of an immunoglobulin disorder are increased sedimentation rate, the presence of rouleaux or immature plasma cells on the peripheral smear, proteinuria, and increased CSF protein without evidence of inflammation.

This test is semiquantitative because, the concentration of the protein is proportional to the amount of precipitate formed in the arc. The patient serum or urine results are compared with the normal control results.

Immunofixation Electrophoresis

Similar to IEP, IFE may be used to detect the presence of immunoglobulins in serum and urine. This procedure is also used to determine if an immunoglobulin disorder exists. In many laboratories, IEP, or IFE is performed, although some laboratories perform both procedures.

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