IMMUNOCHEMICAL TECHNIQUES

(by Lenka Fialová, translated by Jan Pláteník a Martin Vejražka)

Antigens

Antigens are macromolecules of natural or synthetic origin; chemically they consist of various polymers – proteins, polypeptides, polysaccharides or nucleoproteins. Antigens display two essential properties: first, they are able **to evoke a specific immune response**, either cellular or humoral type; and, second, they **specifically interact with products of this immune response**, i.e. antibodies or immunocompetent cells. A **complete antigen – immunogen** – consists of a macromolecule that bears **antigenic determinants (epitopes)** on its surface (Fig. 1). The antigenic determinant (epitope) is a certain group of atoms on the antigen surface that actually interacts with the binding site on the antibody or lymphocyte receptor for the antigen. Number of epitopes on the antigen surface determines its valency.

Low-molecular-weight compound that cannot as such elicit production of antibodies, but is able to react specifically with the products of immune response, is called **hapten (incomplete antigen)**.

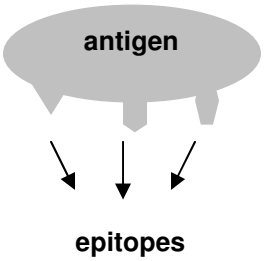


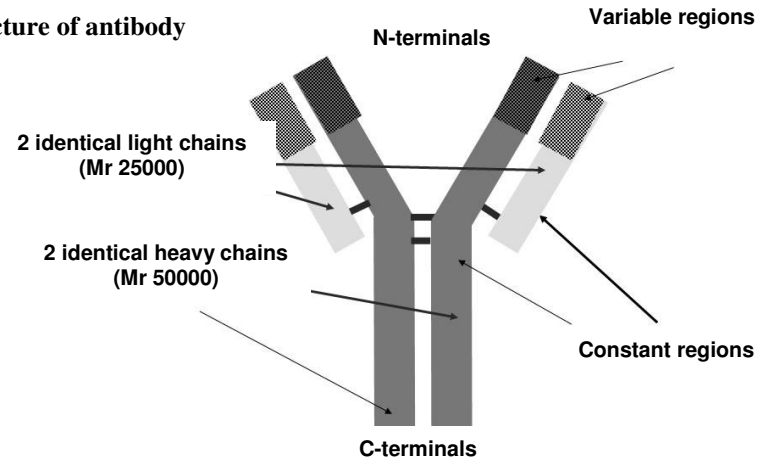
Fig. 1. Antigen and epitopes

Antibodies

Antibodies are produced by plasma cells that result from differentiation of B lymphocytes following stimulation with antigen.

Antibodies are heterogeneous group of animal glycoproteins with electrophoretic mobility β - γ, and are also called **immunoglobulins (Ig)**. Every immunoglobulin molecule contains at least two light (L) and two heavy (H) chains connected with disulphidic bridges (Fig. 2). One antibody molecule contains only one type of light as well as heavy chain. There are two types of light chains - κ and λ - that determine **type of immunoglobulin** molecule; while heavy chains exist in 5 isotypes - γ, μ, α, δ, ε; and determine **class of immunoglobulins** - IgG, IgM, IgA, IgD and IgE. The C terminal parts of both chains form constant regions on an antibody molecule, while the N-terminal parts are designated as variable regions and constitute the portion of antibody molecule that binds antigen – **binding site or paratope**. Except for IgM that has 10 binding sites and IgA with 4 binding sites, immunoglobulin molecules possess 2 antigen binding sites.

Fig. 2. Structure of antibody of IgG class



Immunochemical techniques

Immunochemical techniques are based on a reaction of antigen with antibody, or more exactly, on a reaction of an antigenic determinants with the binding site of the antibody. The antibodies used are produced by various ways.

Monoclonal antibodies are products of a single clone of plasma cells derived from B-lymphocytes, prepared in the laboratory by hybridoma technology, based on cellular fusion of tumour (myeloma) cells with splenic lymphocytes of immunised mice. Monoclonal antibodies are directed against single epitope; and are all identical copies of immunoglobulin molecule with the same primary structure and specificity of antigen binding site. They typically display excellent specificity, but poor ability to precipitate antigen.

Polyclonal antibodies (conventional antibodies) are prepared by immunisation of animals (rabbits, goats, sheep) with the antigen. Blood serum of the immunised animal that contains antibodies against the antigen used, is called an **antiserum**. If one antigen (e.g. one protein) is used for immunisation, **monospecific antibodies** (antiserum) result. However, as every epitope stimulates different clone of B cells, and complex antigens bear several epitopes, the antiserum contains mixture of monoclonal antibodies, differing in their affinity and specificity towards particular epitopes on the antigen used for immunisation.

Immunisation of an animal with mixture of antigens results in production of **polyspecific antibodies**[1], containing immunoglobulins against many antigens (e.g. antiserum against human serum proteins used in immunoelectrophoresis).

Quantitative precipitin curve

Measurement of antigen-antibody complex formation has proved extremely useful for analysing many constituents of body fluids. A wide variety of immunochemical methods has been developed based on the fundamental principle of quantitative precipitin curve described by Heidelberger and Kendall in 1935.

A soluble antigen possessing multiple antigenic determinants reacts with the corresponding antibody and the resulting antigen-antibody complex precipitates out of solution. The precipitin curve describes the relationship between the antigen concentration and the amount of precipitate for a constant quantity of antibody.

Three zones can be distinguished on the precipitin curve (see Fig. 3):

The antibody excess zone: The amount of precipitate increases proportionally as the concentration of antigen increases. If the antibody is present in an excess, all the antigen binding sites are covered with the antibody and only small soluble antigen-antibody complexes are formed. No free antigen is detected in the supernatant, but free (unbound) antibodies can be found. Such conditions are useful for ***immunoturbidimetry, immunonephelometry*** and ***non-competitive immunoassays***.

The equivalence zone: Molecules of antigen and antibody are cross-linked forming large, insoluble complexes. The complexes further aggregate and precipitate. Neither free antigen nor free antibody can be detected in the supernatant. Equivalence is reached in ***immunodiffusion techniques***.

The antigen excess zone: The amount of precipitate decreases due to the high antigen concentration. Large aggregated immunocomplexes decay. As all the antibody sites are saturated by antigen, small soluble complexes prevail. No free antibody but an increasing amount of free antigen may be found in the liquid phase. Excess of free antigen is required for ***competitive immunoassays***.

[1] Polyspecific antibodies are sometimes incorrectly denoted as “polyvalent” and monospecific as “monovalent”. The term “valence” does not, however, correspond to the number of binding sites of the antibody.

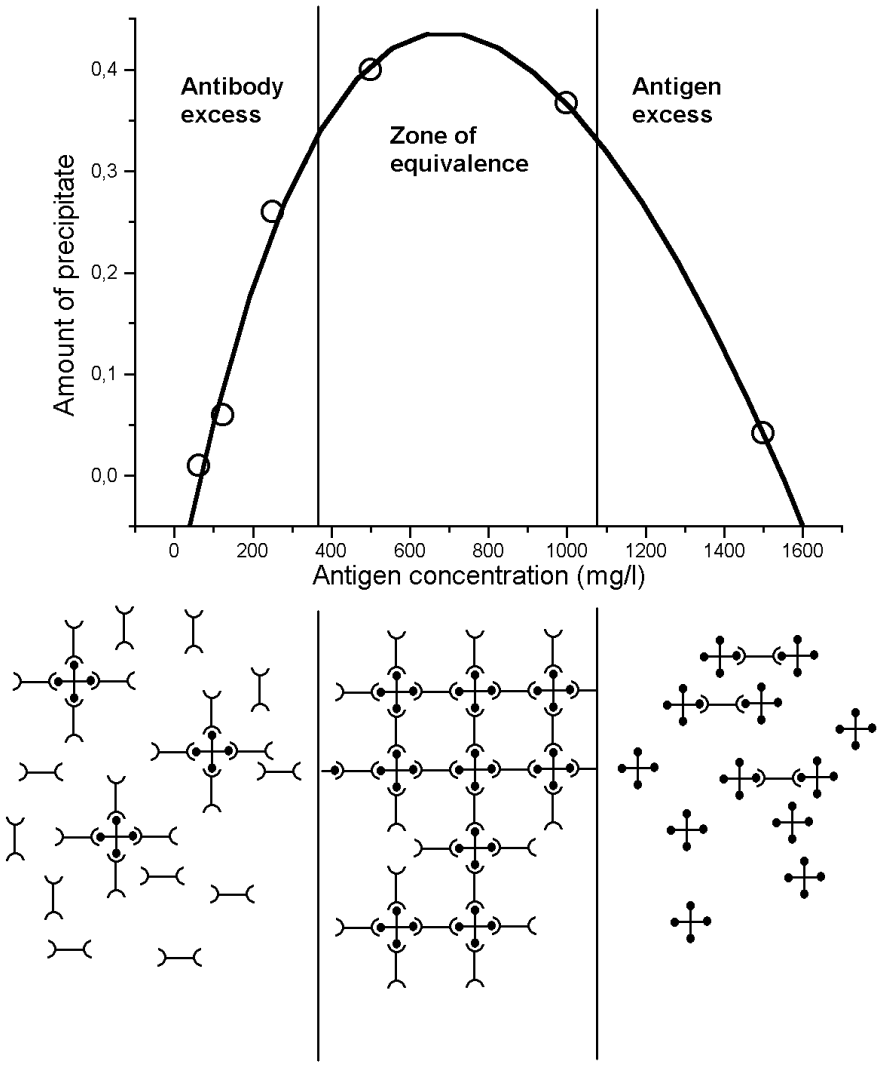


Fig. 3. Precipitin curve

This immunoprecipitin curve forms the basis of many immunochemical assays that can be performed in a solution as well as in a gel.

Precipitation methods in gel

As a support matrix, **agar** or **agarose** gels are used most often. In **single immunodiffusion** only one component (i.e. antigen or antibody) diffuses from the place of sample application, while the other reaction partner is dispersed evenly in the gel. If both components of the immunochemical reaction diffuse in the gel against each other from places of their application, the technique is called **double immunodiffusion**. In the area of antigen-antibody reaction a precipitation zone appears as line, crescent or circle. The immunodiffusion methods in gel are represented e.g. by the **Mancini's single radial immunodiffusion** and the **Ouchterlony's double immunodiffusion**. Other techniques combine an immunochemical reaction with electrophoretic separation, such as **immunoelectrophoresis**, **Laurell's (rocket) immunoelectrophoresis,** and **immunofixation**.

Single radial immunodiffusion

Single radial immunodiffusion represents a simple method without requirements for expensive instruments. The antigen is applied into wells that are cut in the agarose gel containing dispersed corresponding monospecific antibody. The agarose plate is incubated at room temperature for 48-72 hours depending on the specific protein in question. The antigen from the sample diffuses out from the wells into the agarose where the antibody concentration is constant. In a distance from the well where antigen concentration is equivalent to the antibody

concentration (i.e., zone of equivalence is reached in terms of the precipitin curve discussed above), the complex antigen-antibody precipitates and appears as a strong white ring around the well. The square of the ring diameter is directly proportional to the antigen concentration.

The protocol involves several steps:

- Boiled agarose is cooled to 50°C and monospecific serum is added to it. The gel is poured onto a glass plate or a Petri dish.
- After the agarose has solidified, round-shaped wells (starts) are cut out. Samples and standards are applied to the wells.
- The plate is incubated in a wet chamber. The antigen diffuses radially to the gel and reacts with antibody.
- Then, the plate is stained in order to visualise the precipitate rings, and dried.
- Diameters of the rings are measured and squared values are calculated. Calibration curve is plotted using standards. Finally, concentration of antigen in samples is read from the plot.

The sensitivity of this technique is about 10-200 mg/L, and therefore it is suitable for estimation of most serum proteins (e.g. immunoglobulin IgG, IgM, IgA, prealbumin, transferrin, α_2-macroglobulin, ceruloplasmin). This technique has been, however, currently largely replaced with immunoturbidimetry or nephelometry (see below).

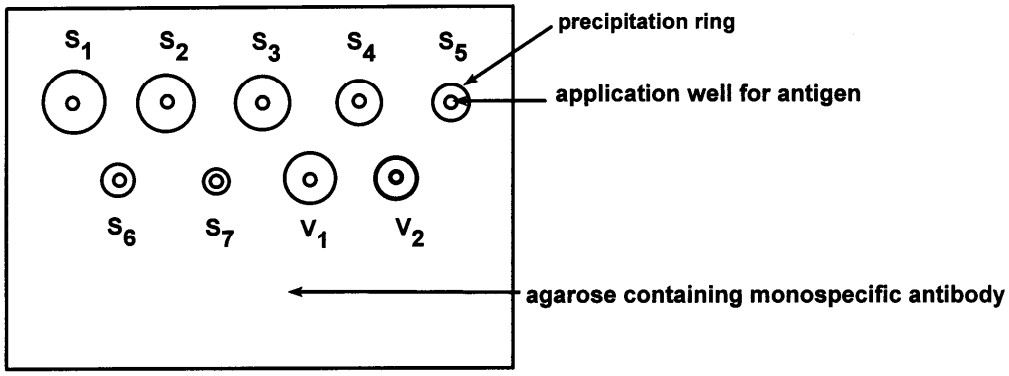


Fig. 4: Single radial imunodiffusion (S_1 – S_7 – standards, V_1-V_2 – samples)

Double immunodiffusion

In double immunodiffusion reaction, the antigen and the antibody diffuse towards each other. The Ouchterlony's modification is the one most often used. It is based on wells punched into the agarose gel in a rosette pattern that are filled with antigen or antibody solutions, respectively. Both antigen and antibody molecules are allowed to diffuse radially into the gel surrounding the wells; and where the antigen and specific reactive antibody meet, a precipitin line forms. If antiserum to several possible antigens is placed into the central well and the outer wells are filled by different antigens, precipitin lines of various shapes can arise. For instance, if two antigenic mixtures are applied into two adjacent wells, the following patterns of precipitin lines can be observed, in dependence on the relationship between the two antigenic mixtures (see fig. 5):

a reaction of identity – if two identical antigens are applied into two adjacent wells, the precipitin bands form a continuous arc.

a reaction of non-identity – the precipitin bands form lines that intersect.

a reaction of partial identity – it is characterised by a formation of a spur. The common hooked precipitation line arises from the reaction of the common antigenic determinants on both antigens with the antibody. The spur means that the second antigen lacks an epitope present in the first antigen that is recognised by one of the antibodies in the antiserum.

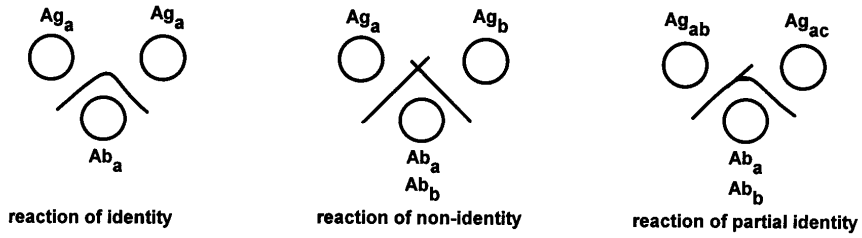


Fig. 5: Double immunodiffusion according to Ouchterlony (Ag_a, Ag_b, Ag_c – antigens with epitopes a, b, c; Ab_a, Ab_b – antibodies against epitopes a and b)

Double immunodiffusion is a qualitative technique suitable for the identification of antigens and the estimation of their mutual immunochemical relationships if the specific antisera are available. On the other hand, this method may be used for characterisation of antibodies using known purified antigens.

Immunoelectrophoresis

Immunoelectrophoresis is a qualitative method that combines protein electrophoresis with immunodiffusion.

It is performed in two steps. The first one involves the separation of antigens according to their charges/size in an electrical field. In the second step, a suitable antiserum (polyspecific or monospecific) is applied to grooves running parallel to the electrophoresis migration zone. The separated antigens and antibodies are allowed to diffuse into the gel towards one another. The precipitation line is formed in the area when the antigen with the reacting antibody meets (Fig. 6).

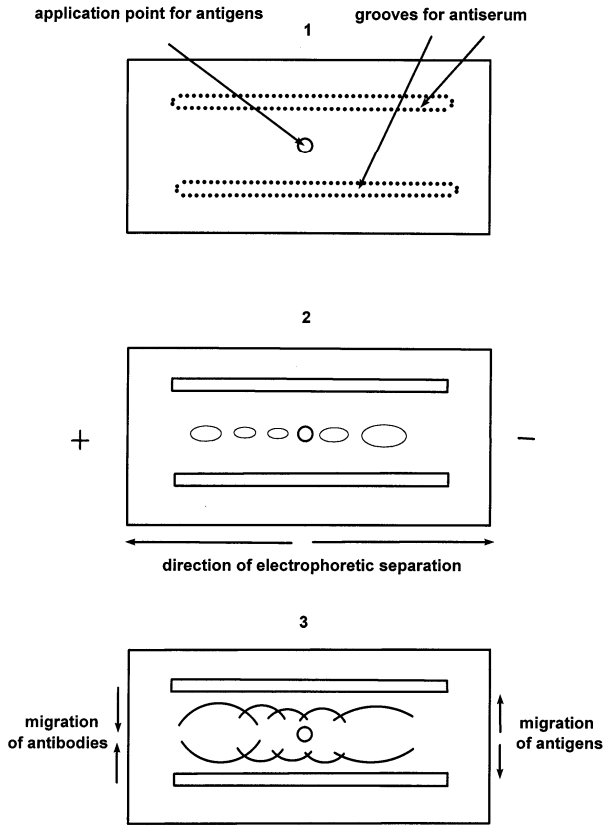


Fig.6: Immunoelectrophoresis (polyspecific antiserum used)

Immunofixation

Immunofixation is a method used for the detection and isotyping of monoclonal immunoglobulins in serum, urine and cerebrospinal fluid. Similarly to immunoelectrophoresis, immunofixation is carried out in two stages. In the first one, serum proteins are separated by electrophoresis. In the second step, the monoclonal immunoglobulins are identified by means of immunoprecipitation with specific antibodies.

The typical procedure of immunofixation is following:
The aliquots of an identical patient serum are applied into six indicated origins in an agarose gel. All the specimens are separated by electrophoresis. At the end of electrophoresis, the gel is covered with the template, in which the areas of the electrophoresis migration zones are cut out. A fixative solution is applied on the first track to denaturate and immobilise the serum protein and to create an electrophoresis reference pattern. The monospecific antibodies, directed against constant regions of IgG, IgM, and IgA heavy chains, and light chains κ and λ, are added to each of the following tracks. The specific antibodies diffuse into the gel. The antigen-antibody complexes are formed in the zone where appropriate antibody and antigen meet. The immunocomplexes are retained in the porous structure of gel and form a marked band, while all the non-precipited components are subsequently removed by washing. Finally, the gel is stained for protein in order to visualise the formed immunoprecipitates.
Use of appropriately diluted sample is the critical step. It follows from the immunoprecipitin curve that excess of either antigen or antibody leads to decay of the immunocomplex. Thus, if both over-diluted or over-concentrated sample is examined, a false negative result may be obtained.

Clinical application of immunofixation

In clinical practice, immunofixation is employed especially for detection of monoclonal immunoglobulins. This technique has replaced immunoelectrophoresis that was formerly used for the same purpose. Immunofixation is easier to perform, more sensitive, and its evaluation is less complicated.

Finding any abnormal band on the conventional serum protein electrophoresis – so called M-component – should be confirmed and further characterised by immunofixation.

Immunoglobulin molecules are produced by the plasma cells. Every plasma cell clone synthesises only one type of immunoglobulin in terms of antibody specificity; the produced antibody molecules are therefore uniform in their structure and function. Monoclonal immunoglobulins (paraproteins) may be in form of polymers, monomers or fragments of immunoglobulin molecules. The finding of monoclonal immunoglobulins indicates an extreme uncontrolled proliferation of a B-cell clone producing Ig molecules. The clinical conditions associated with monoclonal immnunoglubulins - monoclonal ***gammapathies*** – include a wide spectrum from a ***benign form*** to ***the malignant diseases. Multiple myeloma*** (tumour from plasma cells) is the commonest cause of malignant paraproteinemia. Mostly, the myeloma secretes IgG and IgA molecules, which contain only one class of light chain. Occassionally myelomas produce also monomers or dimers of light chains only – ***the Bence-Jones protein***[2]. It is found predominantly in urine, since the light chains have a low molecular weight and therefore, unlike complete Ig molecules, easily pass the glomerular filtration membrane. ***Waldenstrom´s macroglobulinemia*** is another malignancy from plasmatic cells that produces IgM.

Evaluation

Polyclonal immunoglobulins form diffusely stained precipitations detectable in the reaction with one or several heavy or light chain antibodies.

[2] The Bence-Jones protein displays a characteristic atypical denaturation by heat: It precipitates at 50 – 60°C (at pH 4 – 6), at higher temperatures the precipitate dissolves again.

In contrast, the presence of potentially pathologic ***monoclonal immunoglobulin*** is characterised by a narrow homogeneous sharp band within the diffuse immunoprecipitate detected with one of the anti-heavy chain antiserum as well as with one of the anti-light chain antiserum. Usually, one monoclonal protein corresponds to one type of heavy chain and one type of light chain (Fig. 7). The Bence-Jones protein (light chains only) forms a narrow band in a precipitate produced by one of the light chain antisera.

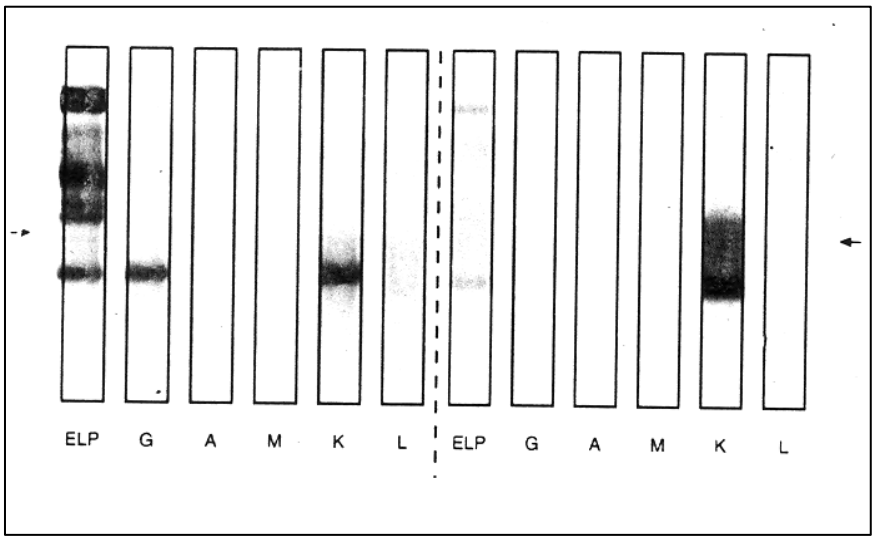


Fig. 7: Immunofixation – example of IgG monoclonal immunoglobulin (serum: IgG κ paraproteins, urine: free light κ chains)

Precipitation methods in solution

The precipitate, which shapes in an agarose gel, can also form in a solution. Two techniques are used to quantitate this precipitate: **immunoturbidimetry** and **immunonephelometry**.

Turbidimetry and nephelometry:
When a diluted antigen solution is combined with a solution of the corresponding antibody, the formation of small aggregates (immunoprecipitates) results in **turbidity** (cloudy appearance) of the solution. Two approaches can be used to quantify this turbidity:

Turbidimetry is based on the measurement of intensity of light transmission; i.e., light that passes through the cuvette is measured. Such measurement can be performed on a conventional spectrophotometer. In contrast, **nephelometry** measures directly the intensity of scattered light**.** Nephelometry requires a special apparatus – the nephelometer, which uses laser as the light source (Fig. 8).

A very critical point in these assays is to work in the **range of the antibody excess** in the reaction mixture, because two different antigen concentrations can in principle give the same value of absorbance – one in the antibody excess and another in the antigen excess zone (see the precipitation curve). This can easily lead to erroneous results. The conventional way of checking whether the measured absorbance lies within the antibody excess or antigen excess zone of precipitin curve is to repeat the measurement with a higher dilution of the sample. With a new sophisticated equipment we can measure the **rate** of turbidity formation, which is directly proportional to the concentration of antigen.

Practical performance of both techniques is simple, and therefore amenable for automation. The suitably diluted monospecific antiserum is mixed with diluted sample; after incubation the light scatter or absorbance of light passing the sample is measured.

Sensitivity of these techniques can be increased by **binding of antibody or antigen onto latex particles**, which considerably increase light scatter due to immunocomplex formation.

The immunoprecipitation techniques in solution are much faster than radial immunodiffusion, but also more expensive.

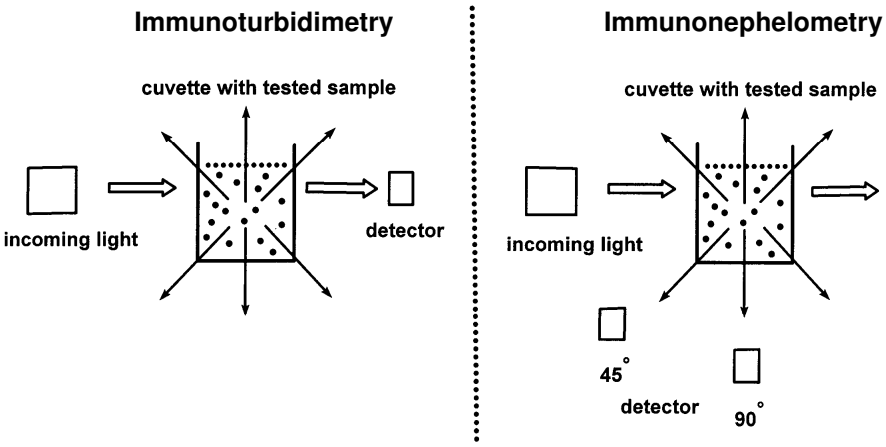


Fig. 8: Immunoturbidimetry and immunonephelometry

Agglutination

Agglutination (from latin 'agglutino' – to glue, to attach) is an immunochemical technique in which a specific antibody reacts with the ***corpuscular antigen***.

Agglutination reaction is based on the formation of bridges between bivalent (IgG) or multivalent (IgM) antibodies and antigenic particles with multiple epitopes. Bivalency or multivalency of the used antibody and multiple antigenic determinants on the surface of particles are necessary for the creation of cross-linking and the formation of a high-molecular-weight lattice that is observable macroscopically. IgM antibodies with ten antigenic-combining sites permit a more effective bridging than IgG. Some antibodies react with the corpuscular antigen, but may not produce agglutination. In this case, the agglutination may be achieved if an anti-immunoglobulin is added into the reaction mixture (the Coombs test).

Hemagglutination is a variant of agglutination technique in which red blood cells are used as the antigen-bearing particles.

Agglutination reactions are performed on slides, in test tubes or microtiter plates. They are more sensitive in comparison with immunoprecipitation methods. The agglutination methods produce qualitative or semiquantitative results.

Agglutination assays may be classified as ***direct*** or ***indirect tests***.

Direct agglutination

In a direct agglutination test, the antigen is an integral part of the cell surface (red blood cells, bacteria). A suspension of particles is directly agglutinated by specific antibodies present in the examined sample. This assay is frequently used in the hematology for the ***determination of blood group*** or in the immunological diagnostics for detection of specific antibodies directed against naturally occurring antigens on the surface of some microbes (for example against *Salmonella typhi* – the ***Widal test***). For the examination of antibodies, the test is usually performed with serial dilutions of the sample. The highest dilution of serum that still causes agglutination is denoted as a ***titer of the antibody***.

Indirect agglutination

Indirect agglutination assay utilises particles with the antigens that have been passively attached to their surface. Originally, red blood cells were used as carriers for antigens; lately, inert particles such as latex, colloid gold and other substances have been shown to be more versatile for the agglutination technique. Many proteins, bacterial and viral antigens are easily adsorbed onto the particle, while other substances require modification by tannic acid or chromium chloride.

The particles coated with the specific antigens are used ***for the detection of antibodies*** against surface antigens in some pathogens and some autoantibodies (e.g. rheumatoid factor[3]) (Fig. 9). Instead of the antigens, particles can also be coated by specific antibodies. This technique is called ***reverse agglutination*** and can be utilised ***for the detection of soluble antigens*** (for example C-reactive protein or human chorionic gonadotrophin) (Fig. 10).

[3] Rheumatoid factors are anti-immunoglobulin antibodies directed against Fc-fragment of IgG. They are usually IgM class antibodies. Increased levels of rheumatoid factors are typical for rheumatic diseases but can be found also in other disorders. Latex particles coated with human IgG are employed for the rheumatoid factor detection (latex fixation test).

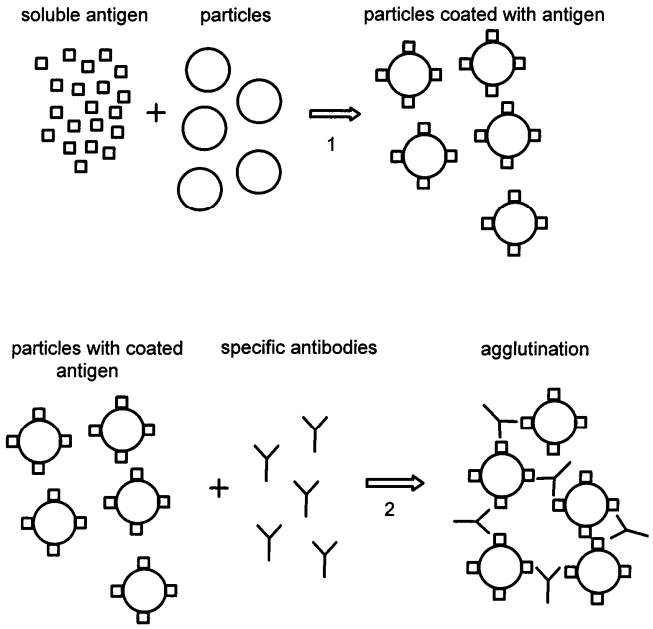


Fig. 9: Indirect agglutination for the determination of antibodies
1st reaction – antigen is adsorbed onto the surface of inert particles
2nd reaction – particles coated with antigens are agglutinated by specific antibodies present in the sample

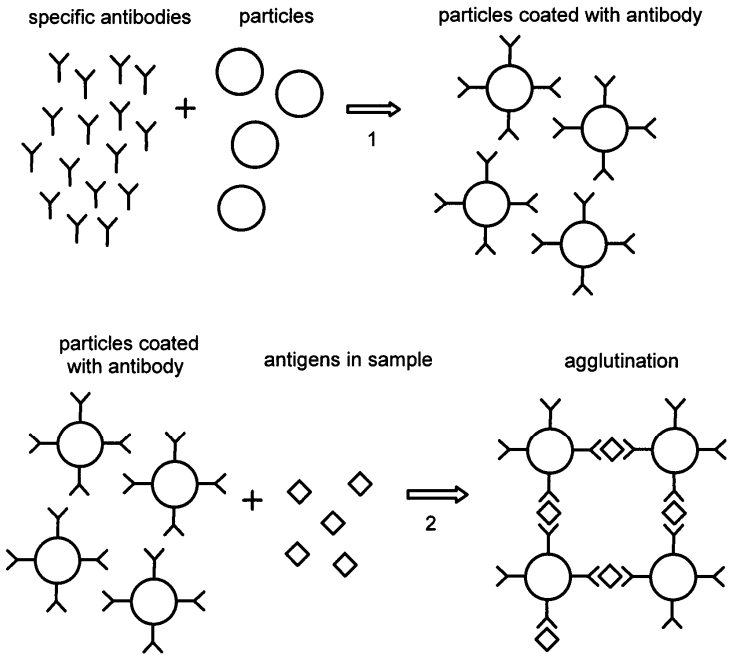


Fig. 10: Indirect agglutination for the determination of antigens
1st reaction – antibodies are adsorbed onto the surface of inert particles
2nd reaction – particles coated with antibodies are agglutinated by the corresponding antigens present in the sample

Agglutination inhibition test

Agglutination inhibition test is another form of agglutination reaction that permits the determination of soluble antigens. It is based on the competition between the antigens in solution and the same antigens on the particle surface for limited amount of antibodies. The specific antibodies are incubated with the test solution containing the soluble antigens. Following the addition of the particles coated with the antigens, the agglutination does not occur because most of the antibodies have been already saturated with a soluble form of the same antigen from the sample. Therefore, antibody binding sites are unavailable for bridging the coated particles. A lack of agglutination indicates a positive result (Fig. 11). On the other hand, if the soluble antigen is not present in the tested sample, after the addition of the corpuscular antigen the agglutination develops.

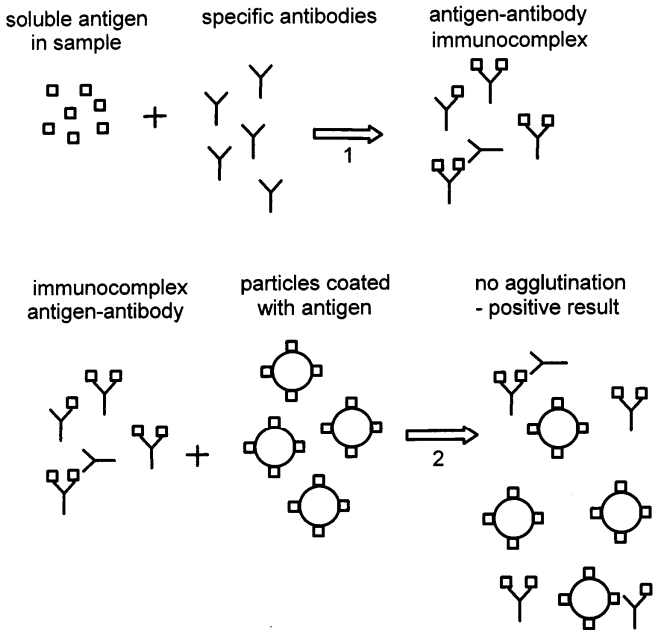


Fig. 11: Agglutination inhibition
1st reaction – a sample containing the soluble antigens is added to the specific antibodies, the antigen-antibody complexes arise
2nd reaction – after the addition of particles coated with antigens, the agglutination does not occur

Enzyme immunoassay

State-of-the-art immunoanalytic techniques achieve high sensitivity by labelling of one reacting component – antibody or antigen – with a substance which detection is more sensitive than detection of immunoprecipitate. The label can be a **radioisotope** (radioimmunoassay, RIA), an **enzyme** (enzyme immunoassay, EIA), a **fluorescent** or **chemiluminiscent** substance. Detection limits of these techniques can be as low as 10^{-15} – 10^{-20} mol/L.

Enzyme immunoassays utilise enzymes, usually peroxidase or alkaline phosphatase, to detect and quantify immunochemical reactions. Both antibodies or antigens can be labelled with an enzyme in order to aid detection.

A **heterogeneous enzyme immunoassay** method is also called **enzyme-linked immunosorbent assay (ELISA)**. In this type of assay, one of the immunochemical reaction components (antigen or antibody) is first non-specifically adsorbed to the surface of a solid phase. Tubes, wells of microtiter plates, and magnetic particles may be used as the solid phases. The solid phase facilitates separation of bound- and free-labelled reactants.

A **homogeneous enzyme immunoassay** is a sort of **enzyme multiplied immunoassay technique (EMIT)** that does not require a separation of bound and free labelled antibodies or antigens. It is simple to perform and has been used for estimation of drugs, hormones and metabolites. Sample containing the estimated antigen is mixed with a known quantity of the same antigen labelled with enzyme (conjugate); and limited amount of specific antibody is added. The unlabelled antigen from the sample competes with the conjugate for the antibody. Binding of antibody on the conjugate results in loss of enzyme activity due to blocking the enzyme active site or change of its conformation. The more unlabelled antigen is present in the solution, the less conjugate will bind to the antibody, and more enzyme activity will be preserved in the solution. Therefore, the enzyme activity is proportional to the antigen concentration in the sample.

The reaction scheme in these immunochemical assays can follow either **competitive**, or **non-competitive** approach.

Competitive enzyme immunoassay

This assay is always performed under condition of **antigen excess**. The enzyme-labelled antigen (conjugate) is mixed with serum sample containing the unknown amount of antigen. The serum antigen and enzyme-labelled antigen **compete for binding sites** of a **limited quantity** of specific antibodies bound to the solid phase. Labelled and non-labelled antigen bind to the antibody in the same proportion as is their proportion in the reaction mixture. In other words, the more non-labelled antigen is contained in the mixture the less labelled antigen is bound (Fig. 12). Under these conditions the probability of the antibody binding the labelled antigen is **inversely proportional** to the concentration of unlabelled antigen. The higher the amount of unlabelled antigen in the sample, the more labelled antigen remains free (unbound). After an incubation, all the unbound both enzyme-labelled and unlabelled antigens are removed by washing along with all other serum constituents. In the subsequent **indicator reaction** for detection of enzyme activity a chromogenic substrate for the enzyme label is added. The intensity of colour is inversely proportional to the concentration of the antigen in serum sample. The results are obtained from a calibration curve constructed with the standards of known concentration of antigen (Fig. 13).

This approach can be used to determine the concentration of small molecules having **only one epitope**, e.g. steroid and thyroid hormones, or drugs in biological fluids.

Non-competitive enzyme immunoassay (sandwich methods)

This kind of enzyme immunoassay can be adopted for measurement of either antigens or antibodies. It is a heterogeneous immunoassay using a solid phase coated with antibody or antigen, which must always be in excess over the analyte being measured.

Non-competitive enzyme immunoassay for determination of antigen:
This non-competitive enzyme immunoassay is suitable for the measurement of large antigens with several antibody-binding sites. Two different molecules of antibodies directed against various epitopes are necessary for performing the assay. The **first antibody** is in excess adsorbed to a solid phase. The serum sample or calibrators containing the desired antigen are added to the well with immobilised antibody. The first imunochemical reaction occurs. Since the antibody is in excess, all antigen molecules should bind. After an incubation, all the non-reacting material in the sample is washed away. Then a **second enzyme-labelled antibody** (different from the first antibody) is added in excess. In the second immunochemical reaction, another antigen epitope binds to the second labelled antibody. "Sandwich complex" consisting of solid-phase antibody – antigen – enzyme-labelled antibody is formed. After washing of all the unreacted enzyme-labelled antibody, the substrate is added. The intensity of the finally measured coloured product of the enzyme reaction is directly proportional to the amount of antigen (Fig. 14).

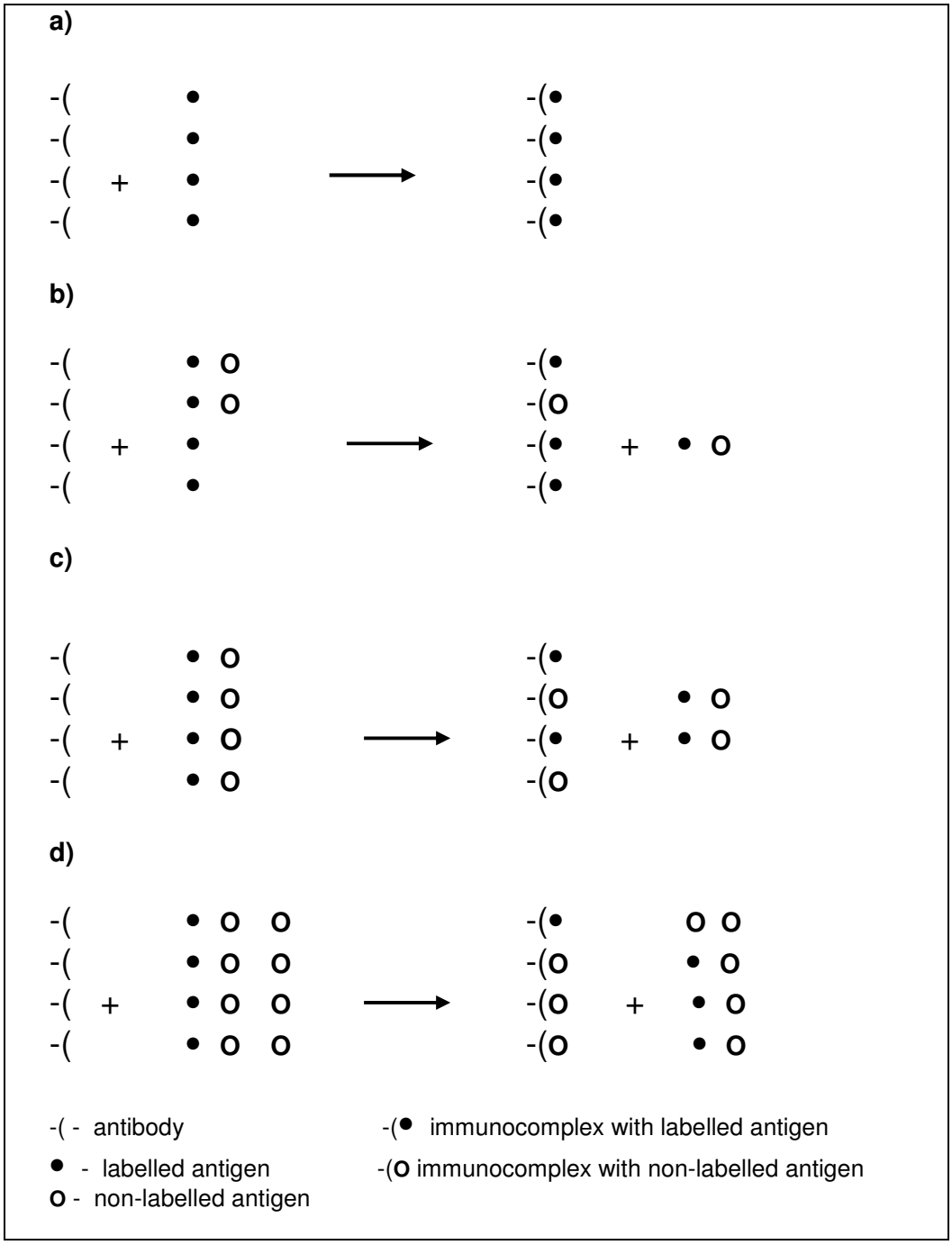


Fig. 12: Principle of competitive immunoassays – various proportions of antigen and antibody

Non-competitive enzyme immunoassay for determination of antibody:
It has been used extensively for detection of serum antibodies to viruses and parasites in serum, autoantibodies, and IgE antibodies specific for a particular allergen. In this case the antigen must be first immobilised on the solid phase. After adding test samples or calibrators the antibodies in the sample react with the immobilised antigen and forms immunocomplexes. The next steps of the assay procedure are similar to that described for the measurement of antigens (Fig. 15).

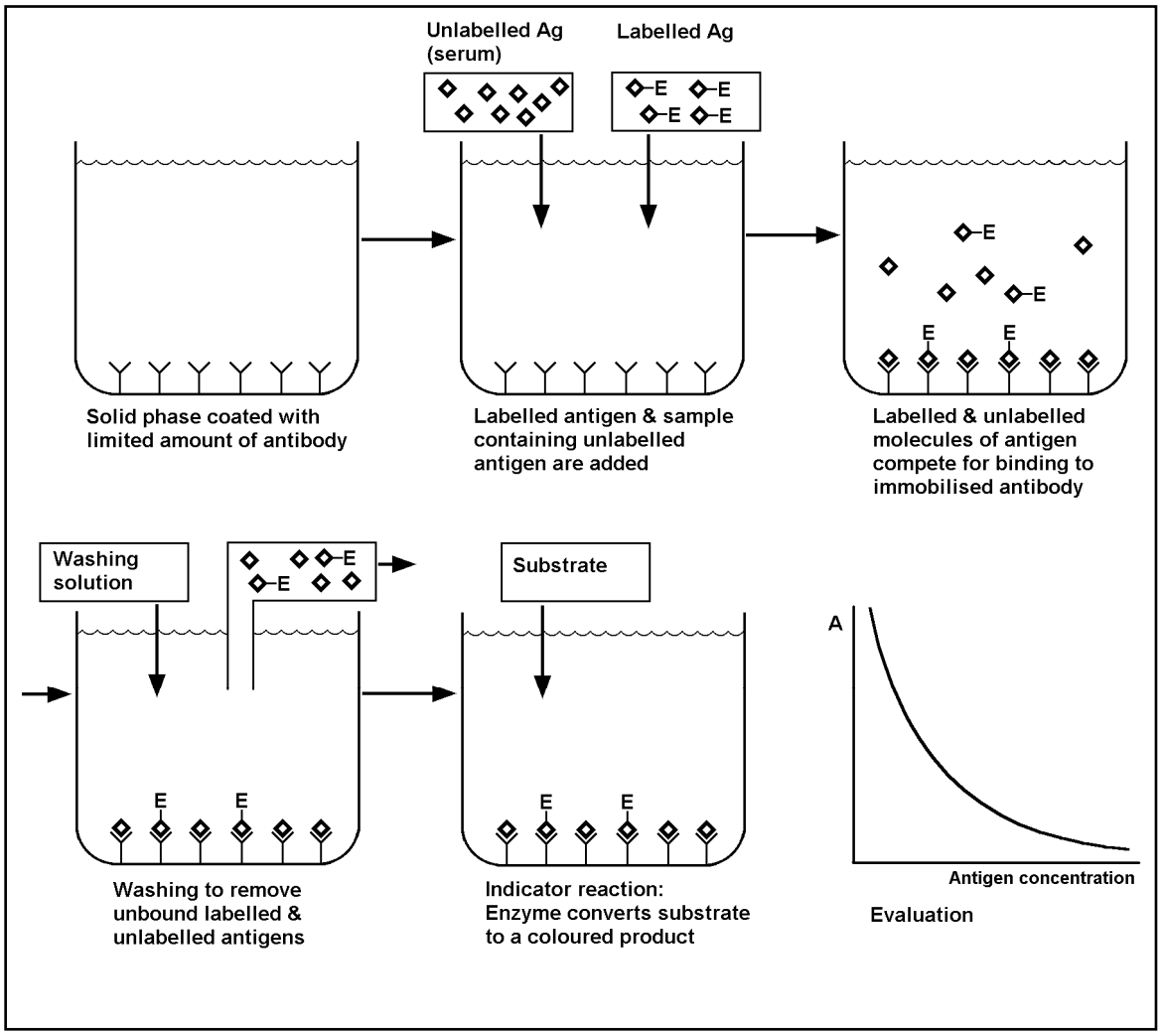


Fig. 13: Competitive enzyme immunoassay

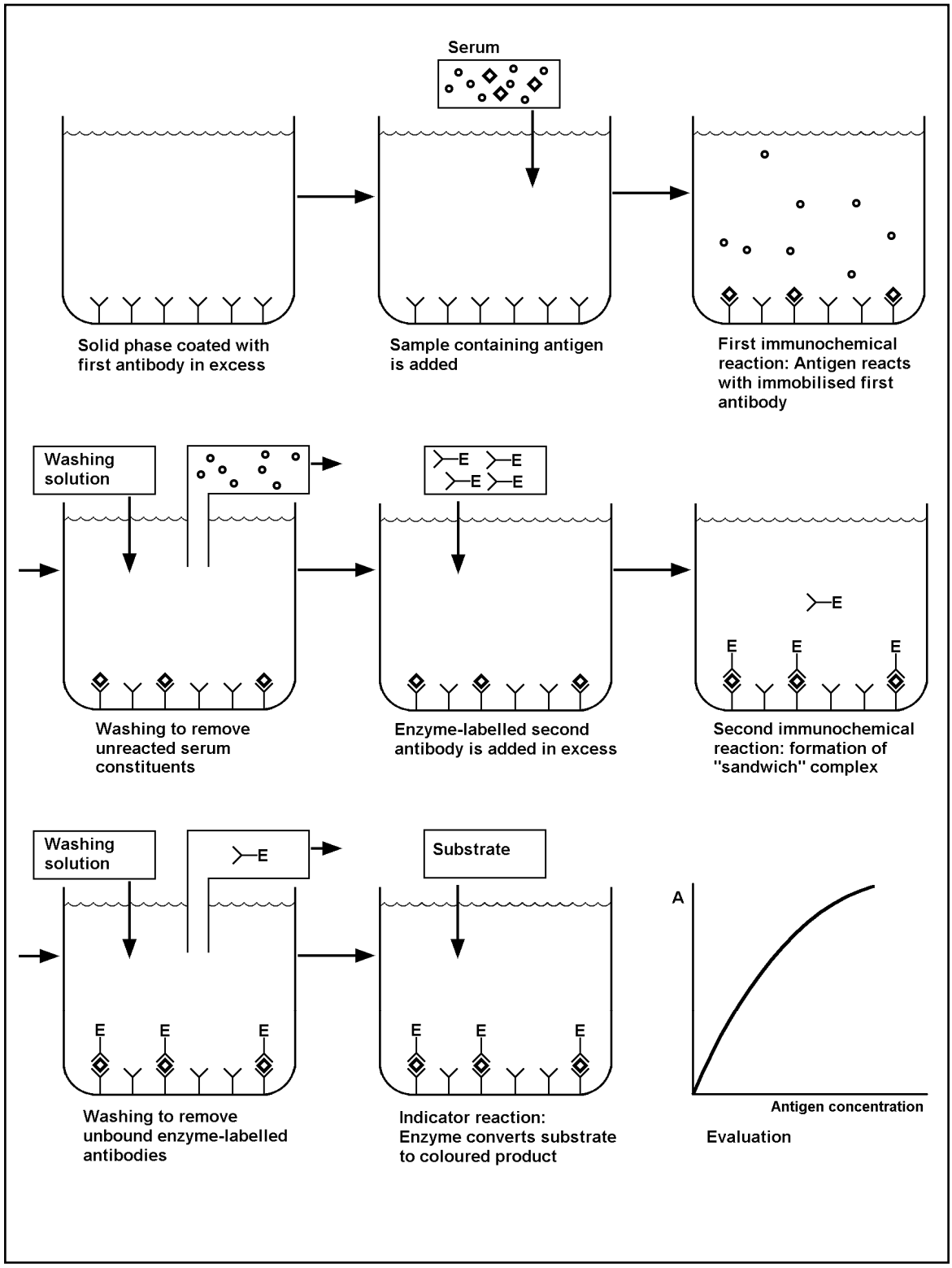


Fig. 14: Non-competitive enzyme immunoassay for determination of antigen

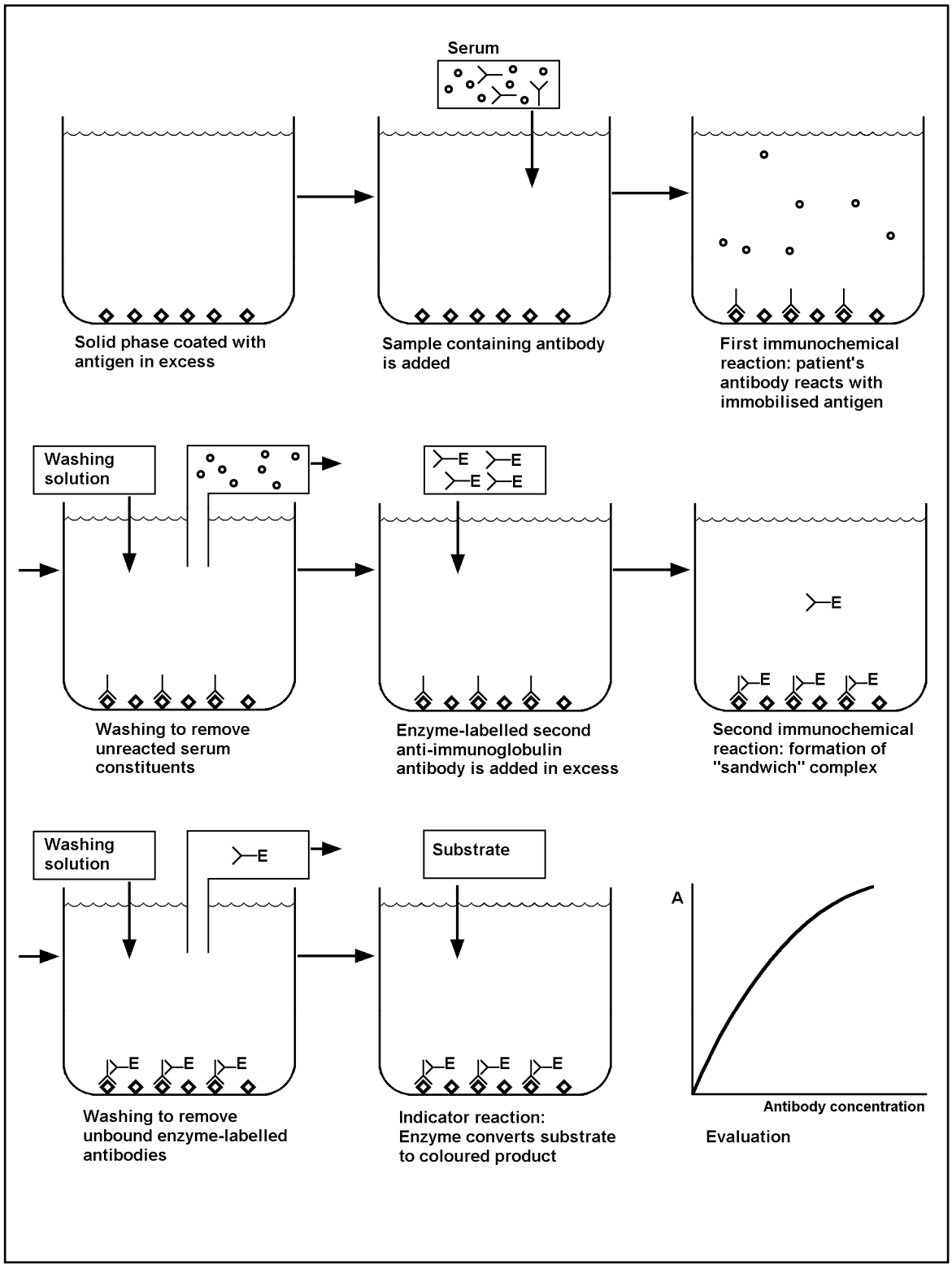


Fig 15: Non-competitive enzyme immunoassay for determination of antibody

Immunochemical methods in fast diagnostics

Fast and tentative "point-of-care" or "bed-side" tests based on "dry chemistry" create another important application of immunochemical methods. Reagents are anchored in a porous substrate that is attached on a plastic pad or inserted in a frame with windows for sample application and result reading. Various setups of tests can be used. Estimation of human chorionic gonadotropin (hCG) for diagnostics of pregnancy, troponine T for acute heart infarction, or tests for narcotics may serve as examples.

Examples of fast immunochemical diagnostic methods

Detection of narcotics in urine or saliva

Tests are based on competition of a drug in the sample with the same drug immobilised in the detection area of the test for a limited amount of specific antibody. The sample is mixed with antibodies in the reaction zone. The antibodies are labelled with stained micro-particles. Then, both the sample and antibodies move by capillary action through the reaction zone to the detection area which contains the immobilised drug. In case the sample contains molecules of the drug it fully saturates the binding sites of colour-labelled antibodies. The antibody molecules cannot then react with the immobilised drug and colour of the detection area stays unchanged. On the other hand if no drug is contained in the sample the binding sites of antibodies remain free and the stained molecules are trapped by the immobilised drug. A coloured band displays in the detection area (Fig. 16).

Tests for individual drugs or for detection of several drugs at once are available. The most frequently abused substances can be estimated by this technique – amphetamine, cocaine, opioids, phencyclidine, and tricyclic antidepressants.

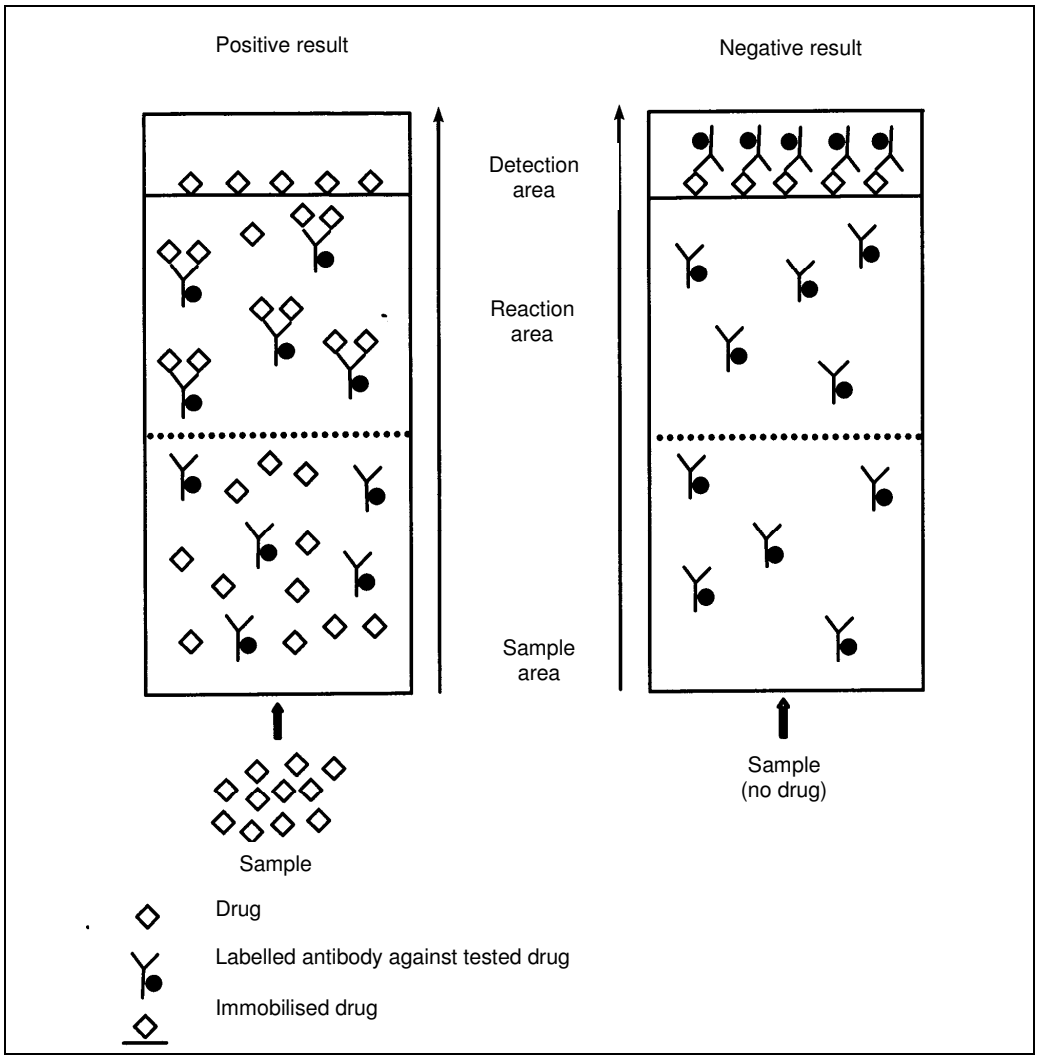


Fig. 16: Tentative test for narcotics in urine

Detection of human chorionic gonadotropin (pregnancy test)

Porous membrane that serves as a support for the test is divided into four areas. Three different antibodies are employed. The first sample area contains specific anti-hCG antibody labelled with microparticles of colloid gold or blue latex (Ab1). When urine sample is applied molecules of this antibody flow to the second, detection area. Another specific antibody against hCG (Ab2) is anchored in this area. If chorionic gonadotropin is contained in the sample a combined immunocomplex with both labelled and anchored antibody is formed (Ab2-hCG-Ab1) and a coloured band displays in the detection area. Excess of the labelled antibody is caught in the third (control) area with immobilised antibodies against labelled anti-hCG. A band in the control area is formed, indicating that the test works properly (Fig. 17). Thus, two bands are interpreted as a positive result.

In case the urine sample contains no hCG the labelled antibody is bound in the third area only. One band in the control zone only is therefore read as a negative result. If no band is displayed in the control area the test is invalid.

Some tests are so designed that the control zone has a shape of a minus sign and the detection zone crosses it perpendicularly. A plus sign therefore appears in case of positive result..

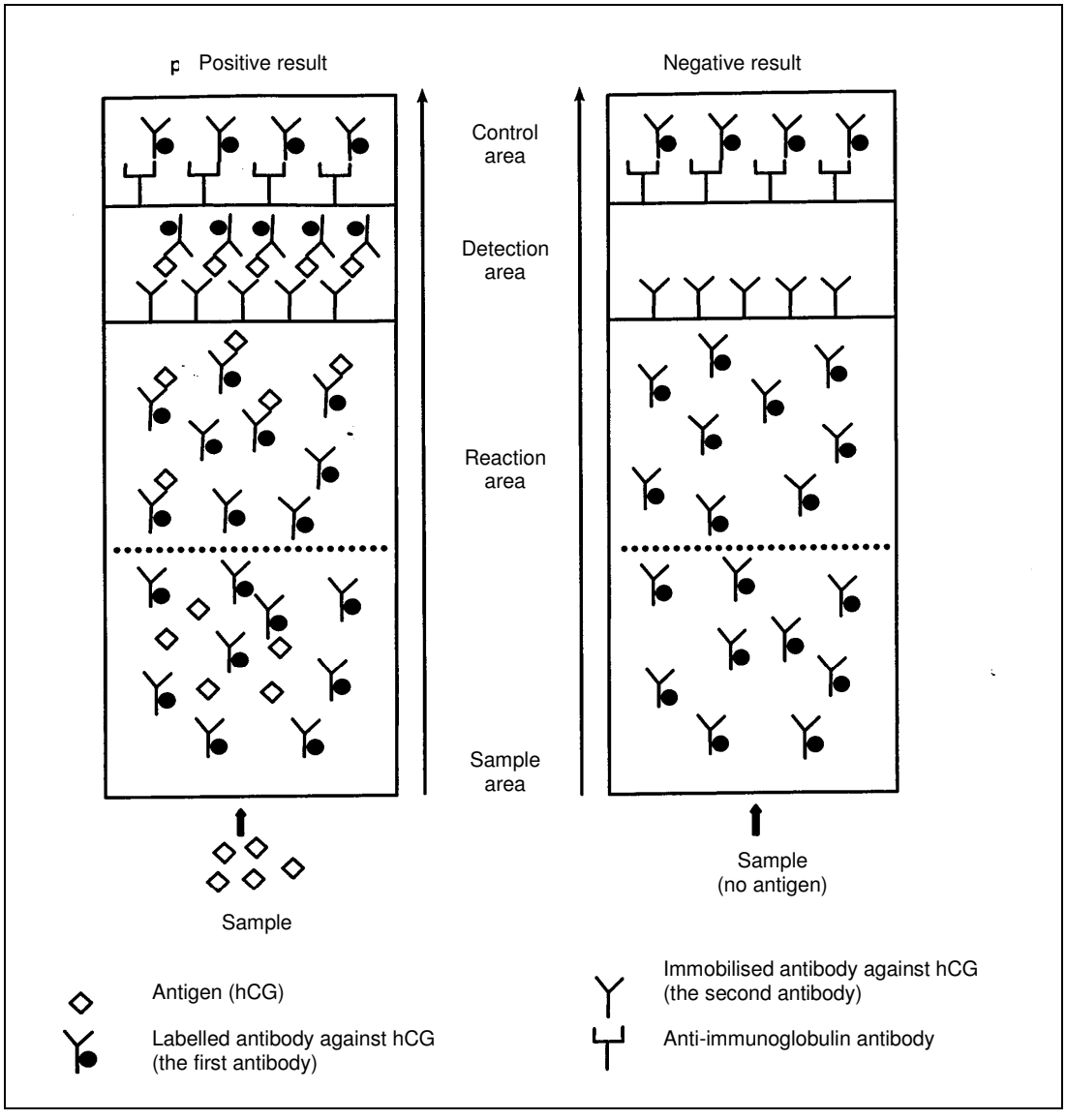


Fig. 17: Tentative test for hCG in urine