Chapter 7 Antigen–Antibody Interaction

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Serologic Reactions for Detection of Antibodies

The union of antibody and antigen gives rise to a series of reactions, the qualitative or semiquantitative study of which is the domain of serology.

The type of reaction observed depends upon the physical state of the antigen (soluble or particulate) and the experimental conditions of the test involved. If the antigen is a soluble protein, the reaction between the macromolecules of the antigen and the antibody, in the proper proportions, results in the formation of an insoluble complex (precipitate). When the antigen is found on the surfaces of particles (such as bacteria or erythrocytes), the molecules of the divalent antibody form bridges with the particles and cause their agglutination (Fig. 7.1). In reactions with erythrocytes, when complement as well as antibody is present, lesion sites form on the erythrocyte membrane through which hemoglobin is liberated. This is the phenomenon of specific hemolysis.

The intensity of a serologic reaction is generally expressed in terms of "titer" – the dilution of serum (or of antigen) in which a specific effect is observed under certain experimental conditions. Thus, for example, if a given serum prepared for experimentation in serial dilutions (1:10, 1:20, 1:40, etc.) produces agglutination at the 1:640 dilution, but does not produce agglutination at 1:1280, it is said to have a titer of 1:640 or to contain 640 agglutinating units per unit of volume. In this type of test, the precision of the reading is obviously subjective and can vary by a factor of $\pm\log 2$ in repeated tests, so that only differences in the titers of two or more tubes of the reaction series are considered significant. In the case of specific hemolysis, however, one can, by using successive dilutions that vary by a factor of less than 2 and through spectrophotometric determination of the quantity of liberated hemoglobin, achieve a precision of $\pm 2\%$.

Although the verification of the intensity of serologic reactions is of inestimable practical value in the diagnosis of infections, it is important to bear in mind that serologic titer does not represent a measure of the

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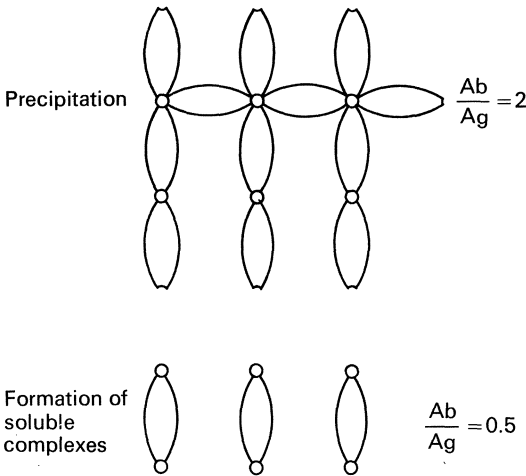


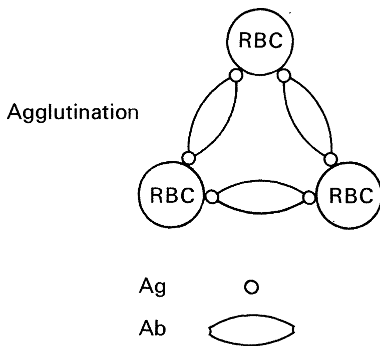


Fig. 7.1. Mechanisms of specific precipitation and agglutination

quantity of antibody, since it depends also upon the quality of the antibody (as in nonagglutinating or noncomplement-fixing antibody) not to mention variations in function from antigen peculiarities and from conditions inherent in the particular test.

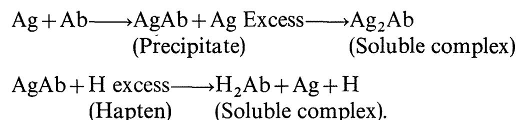
In any case, when comparing the titers of various antisera, one should use the same dosage technique, including, whenever possible, previously standardized reference reagents (sera and antigens).

In Vitro Serologic Reactions

Precipitation

The easiest way of testing the reaction between an antibody and the corresponding antigen in solution consists of layering the two reagents and then observing, at the interface, the appearance of a disk or ring of precipitation (ring test). This reaction, called specific precipitation, occurs whenever the antigenic macromolecule possesses two or three or more combining sites for each of the two combining sites of the bivalent antibody molecule. If the antigen is univalent (hapten) or just bivalent, soluble complexes are formed and a precipitate is not observed (Fig. 7.1). The same occurs when there is an excess of antibody. There is no precipitation when the multivalent antigen reacts with univalent antibody fragments or with antibodies with weak affinity. In the latter case, special methods must be used to detect the presence of the antibody.

The quantitative reactions between antigen and antibody in specific precipitates proceed in variable proportions and are reversible. Accordingly, the antibody (Ab)/antigen (Ag) ratio in the precipitate decreases in proportion to the increase in the quantity of antigen, until a molecular composition of Ag_2Ab is attained, in which soluble complexes are formed:



$$\text{Ag} + \text{Ab} \longrightarrow \underset{\text{(Precipitate)}}{\text{AgAb}} + \text{Ag Excess} \longrightarrow \underset{\text{(Soluble complex)}}{\text{Ag}_2\text{Ab}}$$

$$\text{AgAb} + \underset{\text{(Hapten)}}{\text{H excess}} \longrightarrow \underset{\text{(Soluble complex).}}{\text{H}_2\text{Ab}} + \text{Ag} + \text{H}$$

The latter reaction is the basis of the process for purification of antibodies through the elution of specific precipitates by the corresponding haptens.

Precipitation in Liquid Media. If, instead of layering the antigen and the antibody as in the ring test, aqueous solutions of the two reagents are mixed, initially the mixture is

perfectly clear; after a while, there is a progressively developing turbidity, or opalescence. After a certain period of time, a flocculate, or precipitate, forms that finally collects in the bottom of the tube. The quantity of precipitate is a function of the quantity of antibody present in the antiserum, as well as of the quantity of antigen added. Under these circumstances, if increasing quantities of antigen are added to a series of tubes containing a fixed quantity of antiserum (e.g., 1 ml), an increasing quantity of precipitate is formed, up to a maximum level. Beyond this level, the amount of precipitate diminishes because of the formation of soluble complexes by the antigen excess. By subtracting the quantity of antigen added from the quantity of precipitate formed at the level of maximum precipitation, one obtains the quantity of antibody present in the antiserum.

One can also measure the antibody (or the antigen) in precipitation reactions through determination of the time of the most rapid precipitation, which corresponds to the optimum proportion in which the two reagents combine (optimal proportions method). In the so-called alpha method (Dean and Webb), the concentration of the antiserum is maintained constant whereas that of the antigen is varied; in the beta method (Ramon), varying quantities of antiserum are added to a fixed quantity of antigen. In both methods, the optimum proportion corresponds to that in the tube in which precipitation first appears.

Titration of antibodies by this method is useful in the measurement of antitoxins produced in the horse (Ramon flocculation), whereby determination of the optimum relation is particularly clear because of the presence of precipitation inhibition (formation of soluble complexes) with excess antigen as well as with excess antibody.

Expressed as Lf, the dose of toxin that produces optimum flocculation in the presence of one unit of antitoxin (abbreviated AU) can easily be measured for an antitoxin (A), and a toxin (T), as a function of the relation expressed by the equation

$$\text{ml A} \times \text{AU/ml} = \text{ml T} \times \text{Lf/ml}\,.$$

Thus, for example, if 5 ml of a known toxin (30 Lf/ml) produces optimum flocculation in the presence of 0.1 ml of unknown serum, it can be concluded that such serum possesses 1,500 antitoxin units per milliliter, since

$$\text{AU/ml} = \frac{\text{ml T} \times \text{Lf/ml}}{\text{ml A}} = \frac{5 \times 30}{0.1} = 1\,500.$$

Vice versa, the potency of an unknown toxin that flocculates optimally in a 5-ml dose in the presence of 0.1 ml of a serum titrated at 1 500 AU/ml, can be calculated at 30 Lf/ml as follows:

$$\text{Lf/ml} = \frac{\text{ml A} \times \text{AU/ml}}{\text{ml T}} = \frac{0.1 \times 1\,500}{5} = 30,$$

Gel Precipitation. When an antigen mixture reacts with its antibodies (total antiserum) in a gel medium (agar, Agarose), multiple lines of precipitation are formed that correspond to the specific reactions of each component. It is thus possible by means of gel precipitation to analyse, using various techniques, the components of the antigen mixture (Fig. 7.2).

Simple Immunodiffusion. In this method, introduced by Oudin, the antigen, in aqueous solution, is layered in narrow-bore tubes above a column of 0.6% agar in which the antiserum has first been incorporated. As the solution diffuses through the gel, the antigenic components react with the antibodies to which they correspond. They thus form, in an excellent gradient, rings of precipitation. The position of these rings depends upon the concentration of the antigen and the time of diffusion. The greater the antigen concentration, the farther removed from the surface of the gel is the ring of precipitation. For a known concentration of antigen, the distance "h" from the ring to

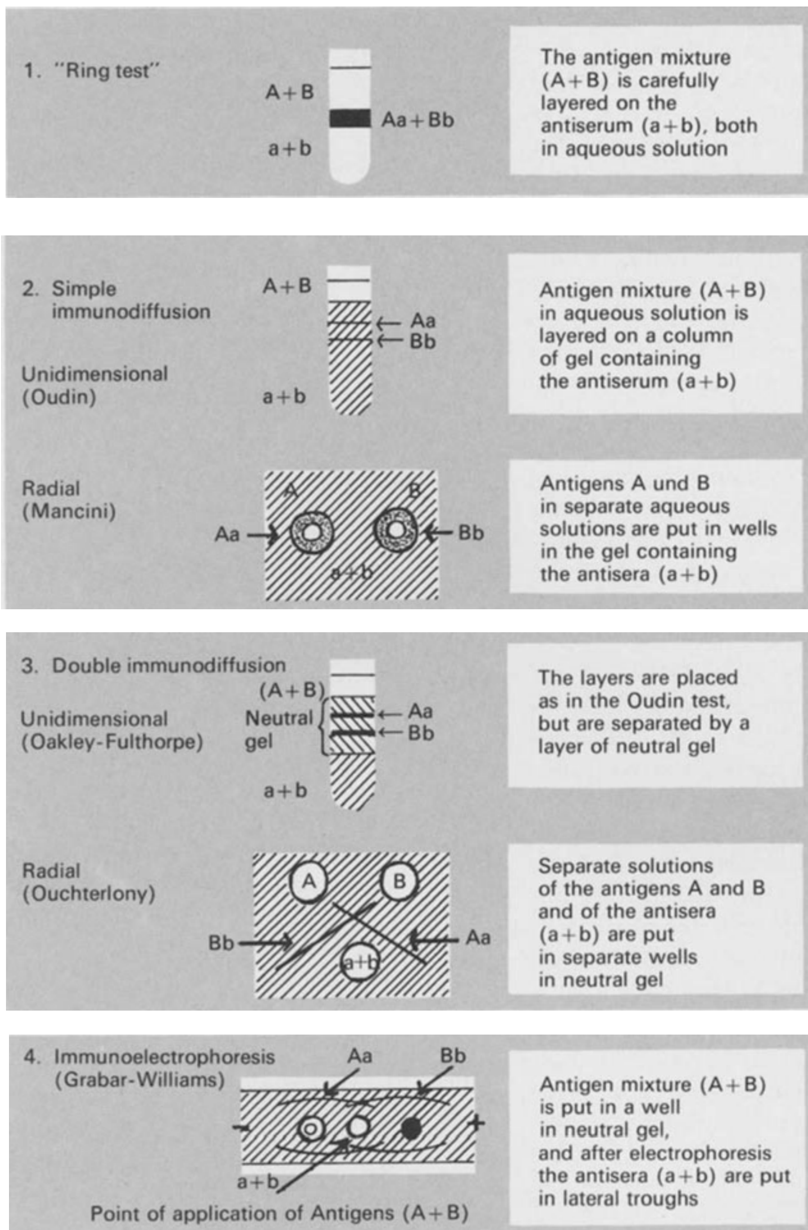


Fig. 7.2. Methods of immunochemical analysis by gel precipitation

the gel–antigen interface is proportional to the square root of the diffusion time, according to Fick's law ($h=k\sqrt{t}$). The distance, therefore, increases after 1, 4, 9, and 16 days by a factor of 1, 2, 3, and 4 respectively; for example, if k = 2, the distance would be 2, 4, 6, and 8 mm after 1, 4, 9, and 16 days.

The specificity of the rings formed with the antigen mixture can be demonstrated clearly with absorption experiments, as exemplified in Fig. 7.3. With Oudin's test, the diffusion in the tube operates in only a single dimension. For the titration of immunoglobulins, however, Mancini introduced simple radial

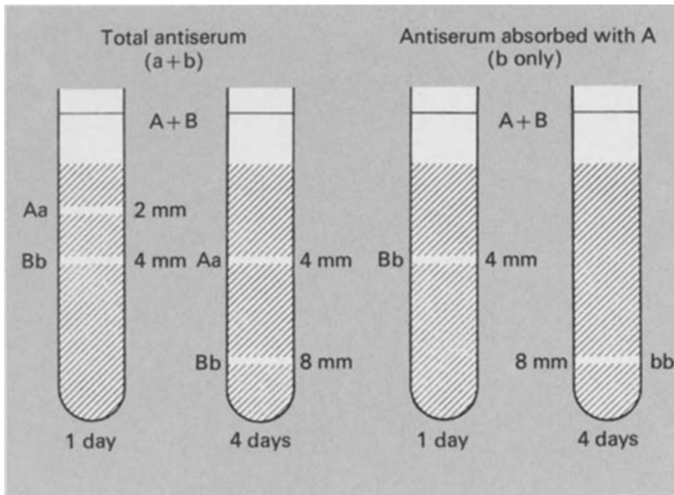


Fig. 7.3. Proof of specificities of the precipitation rings in Oudin's technique through absorption

diffusion[1], in which different dilutions of a standard antigen and of an unknown preparation are placed in separate wells made in a plate of gel in which specific antibody has been incorporated (immunoplate). The antigen diffuses radially, forming rings of precipitation whose diameters are proportional to the logarithm of the antigen concentration. The horizontal distance, m, between the parallel lines obtained with the standard dilution (S) and unknown dilution (D) enables calculation of the relative potency of the latter (Fig. 7.4).

Double Immunodiffusion. Oakley and Fulthrope modified Oudin's technique, interposing a neutral layer of agar between the gel containing the antibody, located in the bottom of the tube, and the aqueous antigen solution, located on the surface. Under these conditions, the antigen diffuses from the bottom upward and the antibody diffuses from the top downward, forming a ring of precipitation in the neutral layer of gel. A variant of this technique (Preer) consists of placing a drop of antiserum in the bottom of the tube, covering it with a layer of neutral gel, and after solidification, adding the antigen solution.

In Oakley's technique, because the diffusion is double, it proceeds in a single dimension. However, in double radial diffusion, introduced by Ouchterlony, the antigen and the antibody – both in aqueous solutions – diffuse into one another from proximate wells cut in a layer of neutral gel. Figure 7.5 illustrates the formation of the precipitin line in the Ouchterlony plate.

With the latter technique, numerous arrangements can be used, depending upon

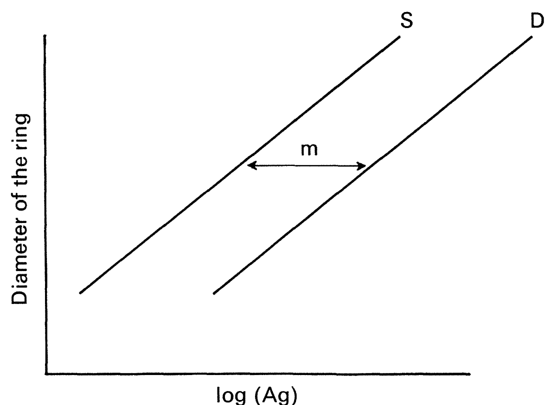


Fig. 7.4. Graph of relative antibody concentration in the Mancini test

1 This is not to be confused with radioimmunodiffusion or radioimmunoelectrophoresis, in which radioactive antigens are used and the precipitation lines are revealed by autoradiography

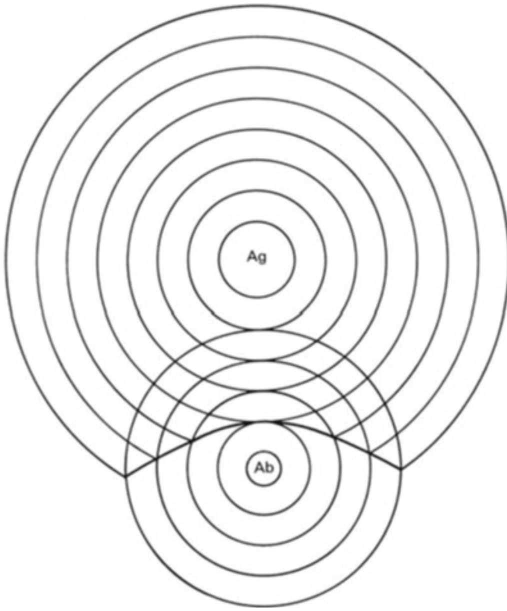


Fig. 7.5. Formation of precipitin line in the Ouchterlony plate

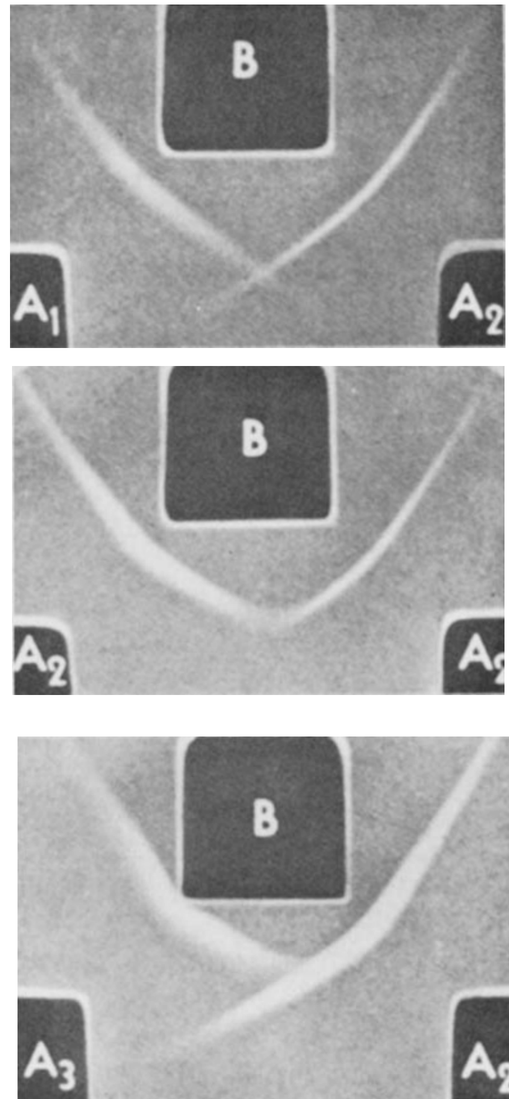
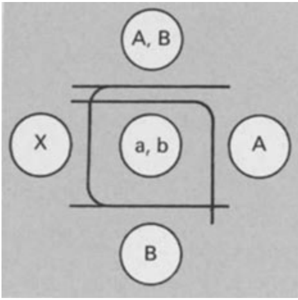


Fig. 7.6. Identical (complete or partial) and nonidentical reactions in double immunodiffusion

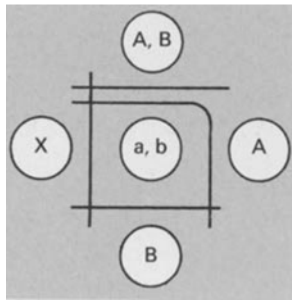
the experimental objectives. One of the most commonly used is that shown in Fig. 7.6. If the front wells contain the same (A 2) antigen, a continuous line of precipitation (identity reaction) is formed. In the case of different antigens (A 1 and A 2) the lines intersect; when there is partial identity (e.g., A 2 and A 1, 2), a spur is formed in the direction of the monospecific antigen (here, A 2).

The use of immunodiffusion methods has permitted detailed analysis of the antigenic components of organic materials. For example, simple diffusion has shown that donkey's milk produces, with a homologous immune serum, five rings of precipitation, three of which disappear when the immune serum is absorbed with horse milk. As many as ten antigenic components have been found in eggwhite, and even after three recrystallizations, chicken ovalbumin disclosed three antigenic impurities. Figure 7.7 illustrates, in diagrammatic form, some examples of antigen analysis with the Ouchterlony test.

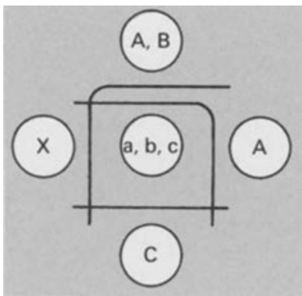
Immunoelectrophoresis. Grabar and Williams combined electrophoresis with immunodiffusion and obtained a better separation of the lines of precipitation. In immunoelectrophoresis, an antigenic mixture is placed in a well in a plate of gel; after electrophoretic separation of the components, the antiserum (total) is added in a longitudinal trough (see Fig. 7.2, 4) along the path of electrophoretic migration.



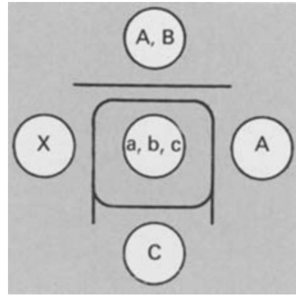
A and B are unrelated antigens; X is partially identical to B, but is not related to A



A, B, and X are unrelated



A, B, and C are unrelated antigens; X is identical to B



A and B are unrelated antigens; A and C exhibit partial identity; X is identical to A

Fig. 7.7. Example of antigenic analysis in Ouchterlony plate

In alkaline pH, the proteins, negatively charged, migrate toward the anode[2], and the components, separated along the axis of electrophoretic migration, diffuse radially to form a series of precipitation arcs with the specific antibodies diffusing from the lateral troughs in the gel.

Immunoelectrophoresis has a considerably greater power of resolution than that of immunodiffusion, making possible the separation of antigenically distinct components that have differing electrophoretic mobilities. For example, in normal human serum, immunoelectrophoretic analysis permits characterization of up to 30 components, instead of five (albumin, α_1, α_2, β, γ) as disclosed by simple paper electrophoresis or agar-gel electrophoresis. In ascending order of their electrophoretic mobilities, the components are differentiated as: γ and β_2 globulins (IgG, IgA, IgM); β_1 (siderophilin or transferrin); hemopexin (β_1B); β_1C–β_1A, β_1E, and β_F, corresponding to C3, C4, and C5; α_2 (haptoglobin, ceruloplasmin, α_2 macroglobulin), α_{1a} (antitrypsin), and albumin (Fig. 7.8).

Two-Dimensional Immunoelectrophoresis. Laurell has developed a new immunoelectrophoretic technique which, besides giving a marked increase in resolution, is especially

2 The electrophoretic mobilities are not determined from the point of application of the sample, but at a point situated to the left, in the direction of the cathode. This is because a current establishes itself in a direction opposite to that of electrophoretic migration. This current, called endosmosis, results from the fact that agar is not completely neutral and possesses an electronegative charge in relation to the buffer in which it is embedded. Under these conditions, since the support-gel is fixed, it is the buffer that moves in the direction of the cathode

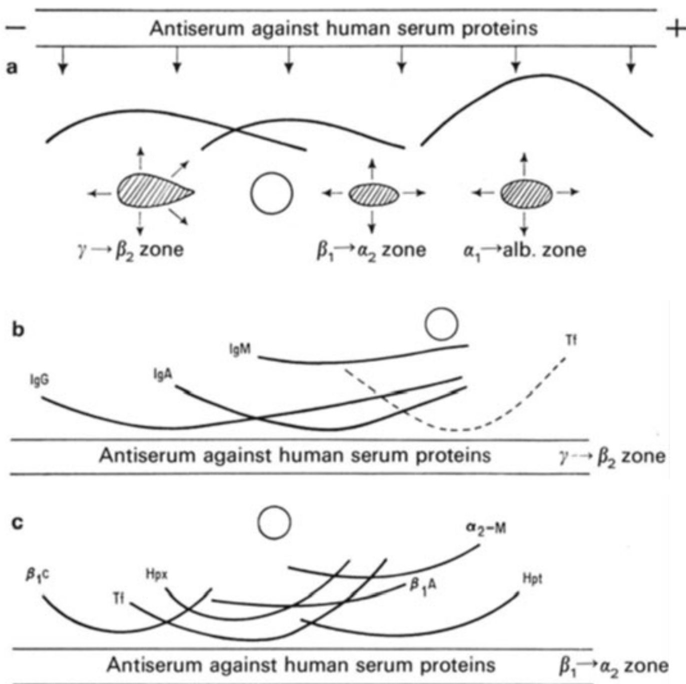


Fig. 7.8. Immunoelectrophoretic separation of the principal protein components of normal human serum

valuable for quantitation. Electrophoresis of the antigen is carried out in one dimension, the gel is cut, unused gel is discarded and warm gel containing the antibody is poured onto the plate. A second electrophoresis is then carried out at a right angle to the initial direction of travel. After diffusion, distinct peaks are obtained whose areas are directly proportional to the concentration of antigen. In the Laurell technique, electrophoretic separation is achieved with a voltage of 10 V/m for at least 60 min (and not at 3–6 V/m as in the technique of Grabar and Williams), so that a cooling device is required.

For the titration of purified antigen the first electrophoretic separation is not necessary. Successive dilutions of the purified antigen are added to contiguous wells of the antibody containing gel, and electrophoresis is carried out at a right angle. As a consequence of the migration of antigen molecules precipitation cones are formed (rather like interplanetary rockets) and the procedure is therefore designated "rocket immunoelectrophoresis".

Counterimmunoelectrophoresis. Another important technique is usually called "counterimmunoelectrophoresis" (a better designation is "immuno-osmophoresis") and consists of cutting wells about 8–10 mm apart in a gel plate. Antigen is added to one well, and antibody to the other. The antigen must migrate more quickly than the antibody at the pH used. After passage of the current, antigen migrates to the anode and antibody, because of endosmotic flow, travels in the opposite direction: $+\overrightarrow{Ab}\ \overleftarrow{Ag}-$. Consequently, the appearance of the precipitation line may occur within 20–30 min, instead of the 24–48 h required in the conventional Ouchterlony technique.

Counter-immunoelectrophoresis has proved to be extremely useful as a routine procedure to detect hepatitis HBs antigen (Australia antigen), in the rapid diagnosis of meningococcal meningitis, etc.

Agglutination

When a suspension of particles that bear antigenic determinants on their surfaces is

mixed with the specific antiserum, large granules are formed that quickly sediment. This is the phenomenon of agglutination, described by Gruber and Durham at the beginning of the century.

The phenomenon may be observed with microbes or cells (erythrocytes, leukocytes, or other cells), in the activity of determinants naturally existing on the surface (direct agglutination), with cells (generally erythrocytes), or inert particles (latex, bentonite, etc.) artifically coated with a soluble antigen (indirect or passive agglutination). In any case, the mechanism of agglutination is fundamentally the same as that of specific precipitation, that is, the formation of bivalent antibody bridges that connect the antigenic determinants of adjacent particles (see Fig. 7.1).

As Bordet demonstrated, the presence of electrolytes constitutes a critical factor in agglutination: In the absence of salts, the particles fix the antibody, but are incapable of agglutinating. This fact caused the authors to espouse a two-stage theory, according to which the union of the antigen and the antibody (first stage) constituted the specific immunologic phenomenon, whereas agglutination represented only a secondary, nonspecific phenomenon (second stage) comparable to the flocculation of hydrophobic colloids by electrolytes. If this concept were accurate, mixing a suspension containing particles of two types, A and B, with an antiserum containing anti-A and anti-B antibodies would result in mixed granules of the two particles. However, in experiments with particles easily differentiated via microscopic examination, such as sheep or chicken erythrocytes, granules were observed containing only one or the other of the particles.

According to the currently accepted explanation, the relevance of salinity in its neutralization of the net negative charge that the particles exhibit in neutral pH, nullifies the repulsion between them and fosters a sufficient degree of attraction. In this way short-range noncovalent forces that assure the binding of the antigen by the antibody are able to determine the formation of bridges between adjacent particles.

Titration of Agglutinating Sera. The agglutinin titer of an antiserum is determined in a semiquantitative test in which decreasing quantities of serum (for example, 0.5-ml dilutions 1:10, 1:20, 1:40, etc) are reacted with a constant quantity of antigen (for example, 0.5 ml of a bacterial suspension containing $0.5–1.0 \times 10^9$ organisms per milliliter). After a period of incubation at the proper temperature, readings of the results are taken, noting the degree of agglutination (+ +, +, −) by the nated eye, or with the aid of a magnifying lens. The agglutination titer is expressed in terms of the greatest dilution that gives rise to complete (+ + +) or partial (+) agglutination. As pointed out previously, the precision of this type of test is only ±50%.

Various factors play an important role in the determination of agglutination titer:

1. The *presence of electrolytes* is essential to the phenomenon, and the pH of the diluent must not be excessively acidic or alkaline in order to avoid nonspecific results. Saline solution is generally utilized as diluent (1.9% NaCl solution), buffered to a pH of 7.2.
2. The *concentration of the antigenic suspension* also constitutes an important factor, for the greater the concentration of particles, the more rapid the agglutination. On the other hand, concentrated antigenic suspensions cause greater consumption of antibody and, consequently, lower agglutination titers.
3. The *temperature* at which the reaction occurs is important. The best temperature for the agglutination of microbes is 37 °C. In hemagglutination, e.g., in the study of the ABO or Rh blood groups, it is convenient to discriminate between the immune antibodies, which react better at 37 °C (warm agglutinins)[3] and the natural antibodies, which agglutinate better at 20 °C; there are also hemagglutinins, such as cryoagglutinins

3 Agglutinin is the term used for agglutinating antibodies

of atypical primary pneumonia or the anti-I agglutinins of acquired hemolytic anemias that react intensely only at 4 °C (cold agglutinins).

4. The *duration of incubation* is also important. One usually takes a first reading of the agglutination test after incubation for 1–2 h at 37 °C and then again after 24-h incubation at room temperature or under refrigeration at 4 °C. Agitation hastens considerably the results in tests performed on plates or on slides with concentrated antigenic suspensions (rapid agglutination). When the test is performed in tubes with diluted antigenic suspensions (slow agglutination), the results can be hastened through moderate centrifugation, followed by gradual resuspension of the sediment.

5. Certain antibodies called *incomplete antibodies* are incapable of agglutinating and, when coexisting with agglutinating antibodies, can block the fixation of the latter and produce a "prozone" of inhibition:

Dilution of the serum	1:10	1:20	1:40	1:80	1:160	1:320	1:640
	–	–	+	+	+	+	–
Agglutination	(Prozone)					(Titer)	

Prozones are observed not uncommonly in certain antibacterial sera such as anti-*Brucella* sera. With anti-Rh antibodies, it is common to observe the exclusive occurrence of nonagglutinating antibodies, which may be disclosed for example through the utilization of diluents with high levels of albumin, the trypsinization of red blood cells, or the antiglobulin test.

It was thought at first that the incomplete antibodies were incapable of agglutinating because they were univalent. Today, however, we know that the lack of agglutination of these antibodies is due to an inaccessible location of the antigenic determinants to which they correspond (Fig. 7.9), or to their weak avidity (low association constant).

It is obvious that an agglutination titer, even when determined under standardized conditions, does not denote the total level of antibodies in the serum, but only that of the predominant antibodies. Thus, for example, if A, B, and C antigens exist on the surface of a particle, the agglutination of these particles in the presence of a whole antiserum (antibodies a, b, and c) will be assured, in

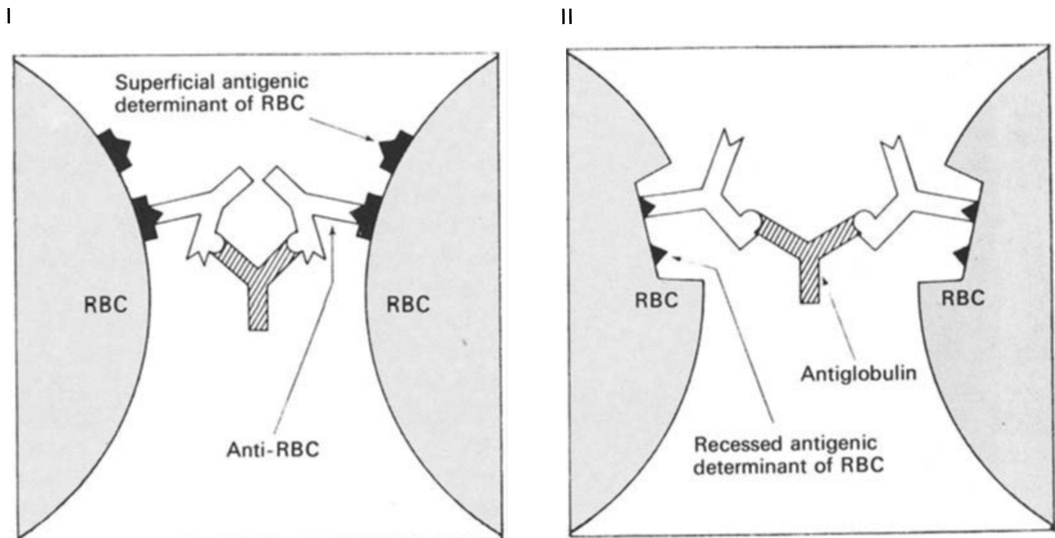


Fig. 7.9. Schematic representation of the absence of hemagglutination observed in certain systems, e.g., in the Rh system. According to interpretation I, the antibody, because it has combining sites that are too close together, cannot unite with two antigenic sites; whereas, according to interpretation II, the antibody is incapable of establishing linking bridges because of the deeply recessed location of the antigenic sites in crypts in the surface of the erythrocyte. In either case, the union can be assured by means of an antiglobulin serum (Coombs' test)

weak dilutions of antiserum, for any of the antibodies –

B-b-B-b-B-b-B
A-a-A-a-A-a-A
C-c-C-c-C-c-C

However, in an end-point dilution (titer), only the molecules of the antibody present in greatest quantity enter into play, e.g., for antibody a –

B B B B
A-a-A-a-A-a-A
C C C C

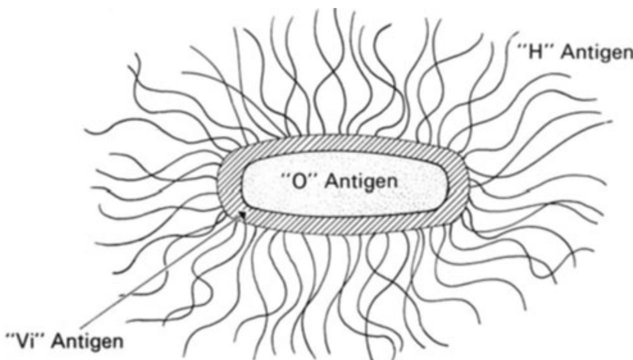
Somatic and Flagellar Agglutination. In motile bacteria such as *Salmonella*, agglutination can be of two types (Fig. 7.10):

1) *Flagellar* (H) *agglutination* occurs when microorganisms unite through their flagella, forming loose floccules that dissociate easily under agitation. This type of agglutination develops rapidly, enabling a reading of the H titer to be taken after 1–2 h of incubation[4].

2) *Somatic* (O) *agglutination* occurs when the union proceeds by way of antibody bridges uniting the bacterial bodies so as to form compact granules, not easily dissociable. This type of agglutination develops slowly, in 24–48 h.

The agglutinogens (antigens that can be detected using agglutinating antibodies) responsible for these two types of reaction can be easily discriminated by heating the bacterial suspension at 100 °C: Such treatment

4 The designations H and O are derived from German and were used primarily for the motile and nonmotile strains of *Proteus*: the former an invasive veil on the surface of the agar, which was compared to breath (Ger. *Hauch*) on a window pane; the other variant, O, grows in isolated colonies, without the invasive veil (Ger. *Ohne Hauch*)



Somatic agglutination	Flagellar agglutination

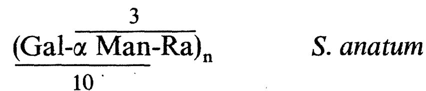
Fig. 7.10. Somatic and flagellar agglutination

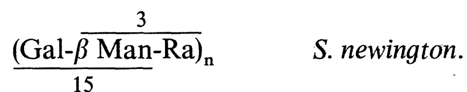
destroys the H antigen without appreciably injuring the O antigen. On the other hand, the agglutinability of the O type is impeded in the presence of 0.5% formalin, which does not act upon H agglutination.
In *Salmonella*, the O antigen is represented by a polysaccharide composed of repeated units (galactose-mannose-ramnose) that – depending upon the mode of linkage among the sugars and/or upon the existence of lateral chains on the basic trisaccharide – exhibit differing specificities (antigenic determinants). As for the flagellar antigens, we know that they are proteins, yet we know nothing about the chemical nature of their determinants.

Cross-Reactions in Microbial Agglutination. When two microbial species exhibit common antigens on their surfaces in addition to their specific antigens, or related antigens, cross-reactions occur among them; that is, the antiserum prepared with the homologous species is capable of agglutinating the heterologous species or vice versa.
We shall not occupy ourselves in this chapter with reactions due to related antigens, which can only be studied properly with the help of precipitation curves. Rather, we shall examine how cross-reactions due to group antigens can be distinguished utilizing the agglutinin absorption test.
Common antigens (group specific) and homologous antigens (type specific) can be represented by distinct molecules or by different regions of the same agglutinogen. In exemplifying this fact, the four species of *Salmonella* represented by the following abbreviated antigenic formulas are considered:

Salmonella paratyphi-B	4/b
Salmonella typhimurium	4/i
Salmonella anatum	3,10/e,h
Salmonella newington	3,15/e,h.

The first two are identical in respect of their somatic antigen (4) but differ in the flagellar antigens (b, i), whereas the last two possess identical flagellar antigens (e, h) but differ in one of the specificities of the O antigen (10 and 15).
If a rabbit is immunized with *S. paratyphi-B*, the total antiserum contains anti-4 + anti-b and, as such, agglutinates *S. paratyphi-B* (4/b) as well as *S. typhimurium* (4/i). The same occurs with the anti-*typhimurium* (anti-4 + anti-i). However, by treating the anti-*paratyphi-B* serum with a thick suspension of *S. typhimurium*, we cause the absorption of the anti-4 agglutinin and thereby produce a monospecific anti-b antiserum.
Vice versa, the absorption of anti-*typhimurium* serum with *S. paratyphi-B* removes anti-4, leaving just anti-i. In this example, the common antigen and the specific antigens correspond to distinct molecules of the two agglutinogens.
The identical result is obtained, however, with the species *S. anatum* and *S. newington*, whose O specificities correspond to distinct regions of the respective agglutinogens:





The 3,10 serum absorbed with 3,15 becomes monospecific anti-10 and, reciprocally, the anti-3,15 serum, absorbed with 3,10, becomes monospecific for 15.
The agglutinin absorption test has been widely utilized by bacteriologists for the differentiation of serologic types – in the enterobacteria group, for example. Utilizing monospecific sera obtained by absorption, more than 1,000 serotypes in the *Salmonella* genus can be distinguished (White-Kaufman table).
The determination of the agglutination titer of sera is important for the diagnosis of infections, as first demonstrated in typhoid fever (Widal's test). The agglutination absorption test permits differentiation of results due to a group reaction or to a mixed infection (Castellani's test). We might illustrate this by a case clinically characterized as ty-

Table 7.1. Model of Castellani's saturation of agglutinins test for the differentiation between group agglutination and mixed infection

Serum of the Patient	0 Agglutination with			
	T	B	T	B
Nonabsorbed	+	+	+	+
Absorbed with T	–	–	–	+
Absorbed with B	+	–	+	+
Interpretation:	Group agglutination		Mixed infection	

phoid fever, in which the serum of the patient exhibited an O agglutination titer of 1:640 for *S. typhosa* (9,12) and of 1:80 for *S. paratyphi-B* (4,12). Castellani's test (Table 7.1) may distinguish the group agglutination in a case of *S. typhosa* infection from that of a mixed infection of *S. typhosa* (T) and *S. paratyphi-B*.

Passive Hemagglutination. It is possible to fix antigens to the surfaces of erythrocytes or inert particles (colloid, latex, bentonite, etc.), making them agglutinable by the respective antibodies. With red blood cells (RBCs), this gives rise to passive or indirect hemagglutination, as opposed to natural or direct hemagglutination, which results from the interaction of antibodies with natural agglutinogens of the RBCs. Various antigens can be fixed simultaneously to the same RBC, which then becomes agglutinable by various antibodies. The same occurs in direct hemagglutination: the anti-RBC serum of sheep, for example, contains a mixture of antibodies with specificities directed against the various natural antigenic determinants of sheep RBCs.

Examples of natural hemagglutination include the agglutination of human erythrocytes by the serum of individuals with different blood groups; the agglutination of sheep erythrocytes by the serum of patients with infectious mononucleosis (Paul-Bunnell reaction); the cryohemagglutination of human O erythrocytes by the serum of patients with primary atypical pneumonia. Examples of passive agglutination include the agglutination of polystyrene (latex) or bentonite particles by the so-called rheumatoid factor; the agglutination of cholesterol crystals coated with cardiolipin by the serum of syphilis patients (Kline and VDRL tests); the passive hemagglutination tests with erythrocytes coated with specific agglutinogens, is utilized in the serodiagnosis of various infections because of their high sensitivity (detection of antibody quantities of the order of 0.003 μg).

Generally speaking, the polysaccharide antigens, when not highly purified [5], can attach directly to erythrocytes. Proteins, however, require prior treatment of the red blood cell with tannic acid (tanning – Boyden's technique), which makes it possible to obtain RBC suspensions specifically agglutinable in the presence of the respective antisera. It is not known for certain how tanning functions; it appears, however, that it not only causes a loose adsorption of the proteins to the red blood cell surfaces, but it also becomes easily agglutinable in the presence of small quantities of antibody. Occasionally, it causes nonspecific agglutination in the control tubes containing no antibody. This last inconvenience generally can be counterbalanced by the use of special diluents (e.g., saline solution with 1% normal rabbit serum).

In addition to tanning, other methods are also available for conjugating proteins to the red cell:

1) *Covalent bonding*. Covalent linkages are achieved by bifunctional molecules such as bidiazotized benzine (BDB), carbodiimide (CDI), glutaraldehyde (GA), and others.
2) *Metallic bridge*. Certain multivalent cations, Cr^{+} in particular, modify the red

5 Fixation of polysaccharides to red blood cells appears to depend upon the presence of ionized sugars (amino sugars, uronic acids). Thus, for example, the O polysaccharides of *Salmonella* obtained by alkaline hydrolysis, which possess the characteristics described previously, attach more easily to erythrocytes than do highly purified polysaccharides obtained through acid hydrolysis

blood cell surface, making it capable of adsorbing proteins.

3. *Immunologic bridge.* To avoid autoagglutinating suspensions because of the conjugation of erythrocytic proteins, one may resort to an interesting method that consists of the following steps: (1) the protein antigen is conjugated by means of BDB with nonagglutinating anti-Rh antibodies; (2) the conjugate is then fixed to Rh-positive red cells (immunologic bridge).

Regardless of the method employed, the specificity of each amount of erythrocyte suspension must be controlled, for small variations in technique can noticeably affect the degree of autoagglutinability. Today there is a tendency to fix the erythrocytes before or after the fixation of the antigen to obtain suspensions that remain unchanged for months when maintained at 4 °C.

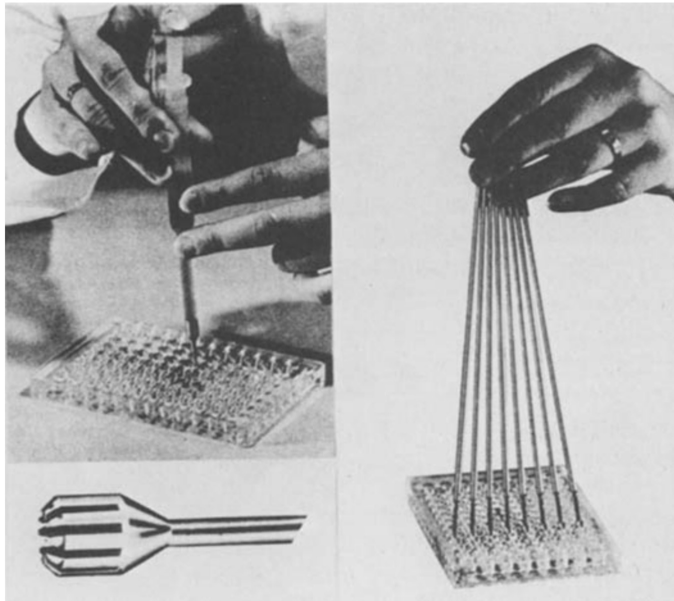
The erythrocytes most commonly used are human erythrocytes or sheep erythrocytes; in certain cases, however, there is an advantage in using erythrocytes from other species.

The quantity of antigen fixed to the erythrocytes is of major importance. It is advisable to determine the optimum quantity in preliminary experiments; that is, the quantity that produces the highest titer in comparison to a reference antiserum. The minimum number of molecules capable of "sensitizing" the erythrocytes can be determined by using antigens labeled with isotopes. The minimum number of molecules of O polysaccharide of *Salmonella* is of the order of 2,000 molecules per erythrocyte.

The hemagglutination reaction itself can be performed in tubes or, more conveniently, on plastic plates with wells in which different dilutions of serum to be tested are distributed along with a constant dose of the erythrocyte suspension. This latter technique is practical because it utilizes only 25 μl of serum. This assemblage, shown in Fig. 7.11, consists of a plastic plate with 75-μl wells and a metal loop titrator with a 25-μl capacity (Takatsy microtitrator). Into each well, 25 μl of diluent is pipetted; then 25 μl of the antiserum under study is deposited in the first well. The microtitrator is then dipped into the first well, and the solution is mixed well by rotating the stem of the microtitrator between the fingers. Then 25 μl of successive dilutions of serum at 1:2, 1:4, 1:8, etc. are passed successively from one well to another. Subsequently, 25 μl of 1% erythrocyte suspension is added, agitating carefully to mix the reagents. The plate is covered or placed in a humid chamber to prevent evaporation and incubated at the desired temperature. Results are generally read after 2 h at room temperature and after 12–24 h when refrigerated at 4 °C. In the tubes as well as in the plates, the results are interpreted in conjunction with the pattern of the sediment, which exhibits the form of a button in negative reactions (−), that of a round plate with irregular borders in strongly positive reactions (4+), and with intermediate patterns in the +, 2+, and 3+ reactions (see Fig. 7.11, bottom). In case of doubt, the reading can be confirmed by gently resuspending the sediment and then observing with a lens the presence and size of the granules.

Immunofluorescence

It is possible to make the antigen–antibody reaction visible by labeling one of the reagents with substances called fluorochromes, which have the capacity to absorb luminous energy, to store it for short periods (10^{-9}–10^{-1} s), and then to emit it in the form of radiation of a greater wavelength. This mechanism of fluorescence is due essentially to the absorption of the energy of photons by electrons of peripheral orbits that move to occupy orbits more distant from the nucleus, inducing a state of excitability in the molecule. Such a state is, however, of extremely short duration, because the electrons quickly return to their former orbits, i.e., to a state of repose, due to the emission of luminous radiation. Because part of it is degraded into thermal or mechanical energy, the quanta of light emitted (fluorescence) have less energy, or greater



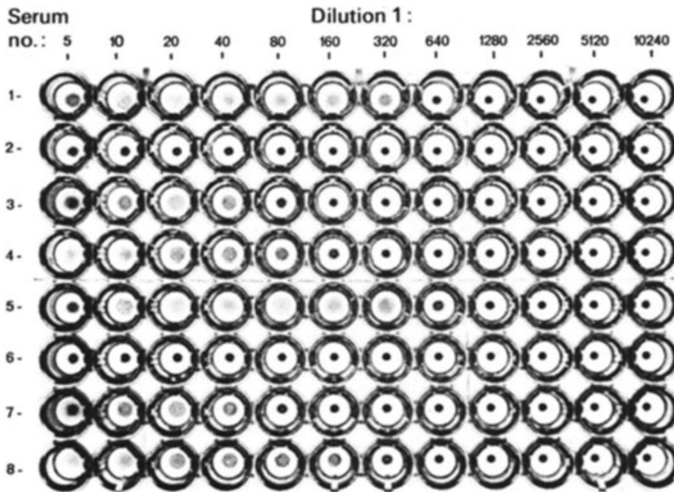


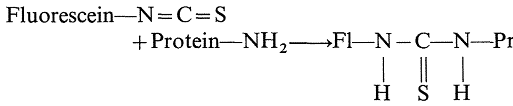
Fig. 7.11. Hemagglutination in the microtiter plate (Takatsy's microtitrator)

wavelength, than that of the exciting radiation (Stokes law).

For this reason, anti-DNA antibodies labeled with fluorescein have an absorption maximum at approximately 490 nm [1 nm (nanometer) or millimicron (mμ) is equal to 10^{-9} m, or 10^{-6} mm], whereas its emission maximum takes place at around 530 nm. If a section or cellular smear is treated with labeled antibody, the cytoplasm appears blue, whereas the nucleus exhibits green-yellow fluorescence.

In addition to fluorescein, rhodamine B, which emits an orange-red fluorescence, is also frequently used. Both fluorochromes are used in the isothiocyanate form, which

conjugates easily to proteins in alkaline pH (>9):



(Isothiocyanate of fluorescein)

For microscopic observation of fluorescence, the following accessories are necessary: (1) a source of excitatory light; (2) a thermal filter; (3) an excitatory filter; (4) a dark-field (cardioid) condenser; and (5) a "barrier" or protector filter. The source of excitatory light is generally in the form of a quartz bulb with mercury vapor (Osram HB 200 lamp), which emits visible and ultraviolet radiation (below 400 nm).

The light proceeding from the excitatory source passes successively through the thermal filter and the excitatory filter – the latter permeable only to radiation of a wavelength around 435 nm, which is still situated in the band of absorption of the fluorescein. Next, the excitatory light is directed at the cardioid condenser, which projects it along the microscope's optical axis on to the preparation. The light transmitted by the preparation includes not only radiation of the same wavelength, but also radiation having a wavelength longer than that of the excitatory light (fluorescent). The barrier filter interposed between the preparation and the eyepiece protects the eye of the observer from short wavelength radiation that passes through the objective (Fig. 7.12).

In principle, two techniques are utilized for the study of immunofluorescence (Fig. 7.13)

1) *Direct immunofluorescence*. This involves direct coloration of the antigen with labeled antibody. It is commonly used to identify microorganisms by immunofluorescence, e.g., *E. coli* (enteropathogenic serotypes), *Klebsiella* (serotypes), *Streptococcus* (Lancefield groups), *Gonococcus*, *B. pertussis*, *C. diphtheriae*, *Leptospira* (serotypes), and *Candida albicans*.

2. *Indirect immunofluorescence*. The specimen with attached antigen is treated with an unlabeled specific antibody and then, after washing, incubated with conjugated anti-gamma globulin produced against the immunoglobulin of the species from which the specific antibody originates.

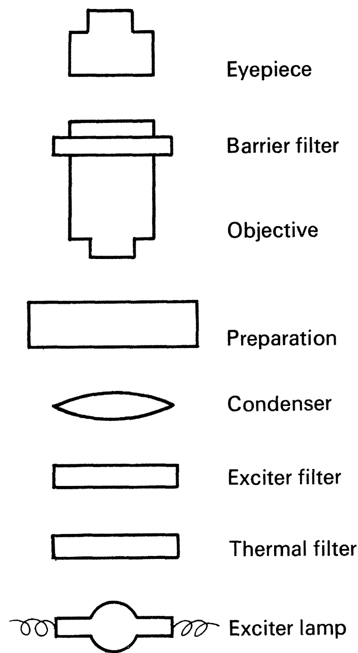


Fig. 7.12. The optical system in fluorescence microscopy

The double-layer immunofluorescence just described is frequently utilized to demonstrate antimicrobial antibodies (serodiagnosis of syphilis, toxoplasmosis, leptospirosis, schistosomes, Chagas' disease, etc.), as well as to detect autoantibodies, e.g., antinuclear antibodies in lupus erythematosus or intercellular antibodies in pemphigus. There can also be formation of triple layers, as in the so-called sandwich technique. The first layer is an antigen–antibody complex, a second layer, unlabeled antibody against antibodies of the first layer, and the third, labeled antibody with a specificity directed against the second layer. This technique is used to demonstrate antibody on the surfaces of plasma cells, as performed by Coons and his associates.

The immunofluorescence reactions for the demonstration of complement fixation are

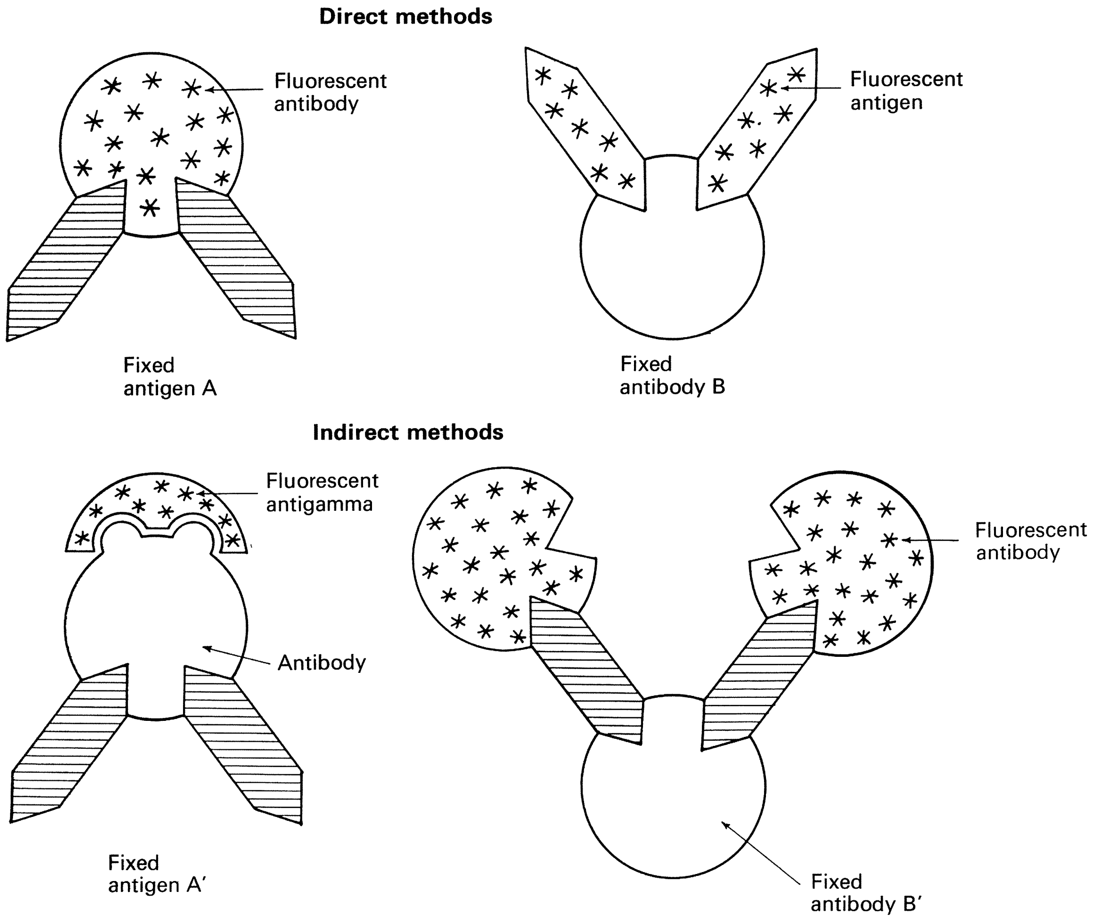


Fig. 7.13. Different methods of immunofluorescence

similar to the sandwich technique. A first layer is composed of an antigen–antibody complex; a layer of complement is adsorbed to it; then a third level of labeled anticomplement is added. [Anticomplement is obtained by immunization with antigen-antibody complex or with zymosan-C3 (production of anti-β^1C serum).] The demonstration of complement fixation in vivo by immunofluorescence suggests that the lesions such as those that occur in the Arthus reaction (vasculitis), in certain forms of glomerulonephritis by the deposition of antigen–antibody complex or by cytotoxic antibodies, are produced by antigen–antibody complexes.

In any case, one must utilize reagents previously characterized as to their activity (titration of the conjugate to determine the optimum dose) and their specificity (absence of nonspecific fluorescence), which generally can be achieved by using conjugates obtained from potent antisera and with discrete labeling (low fluorochrome/protein ratio).

Complement Fixation

In combining with the antigen, certain antibodies affiliated with the IgG and IgM immunoglobulins form complexes capable of fixing complement. The phenomenon was discovered at the beginning of the century by Bordet and Gengou and quickly aroused great interest because of its application in the serodiagnosis of syphilis (Wassermann

reaction). Today, numerous other infections are diagnosed by the complement fixation reaction (CF), e.g., Chagas' disease, South American blastomycosis, toxoplasmosis, echinococcosis, gonococcic infections, rickettsiosis, and numerous virus infections (psittacosis, lymphogranuloma, poliomyelitis, arbovirus infections, epidemic parotitis, influenza). Moreover, the CF test is also utilized, through the use of known antisera, to characterize the types and subtype of numerous viruses, such as the aftosa viruses, the arboviruses, and the echoviruses.

The Qualitative Test. The complement fixation test can be summarized in the following manner:

I. Specific antigen (Ag)+C	Free C
II. Anti-Ag antibody+C	Free C
III. Anti-Ag+Ag+C	Fixed C.

If EA (e.g., sheep erythrocytes sensitized by rabbit antisheep erythrocytes) is added to mixtures I and II, hemolysis occurs:

EA + free C = Hemolysis.

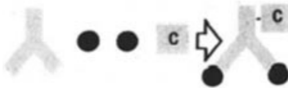
The addition of EA to mixture III, however, produces little or no hemolysis, for part or all of the complement mixed with the Ag-anti-Ag complex will have been consumed:

EA + fixed C $\rightarrow$ Absence of hemolysis.

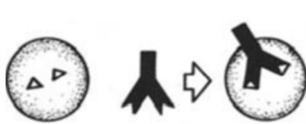
The qualitative test can be limited to the three tubes mentioned previously – I and II being antigen and serum controls, respectively, and III being the reaction tube. The mechanism of the test, in its two stages, is represented schematically in Fig. 7.14.

In the early days of serology, when the Wassermann reaction was introduced, the degree of fixation was evaluated by the percentage of hemolysis observed, expressing the results as + + + + (absence of hemolysis), + + + (25% lysis), + + (50% lysis), and + (75% lysis). Today, however, to evaluate

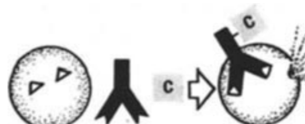
Positive reaction



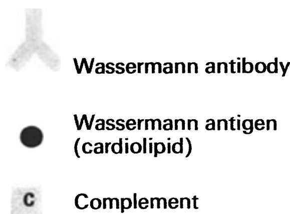
Negative reaction



No hemolysis



Hemolysis



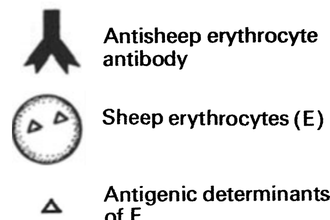


Fig. 7.14. Mechanism of the Wassermann reaction for complement fixation

the fixative potency of a serum, increasing dilutions of antiserum are mixed with adequate fixed amounts of antigen and complement, proceeding to a reading of the results in terms of the quantity of hemoglobin liberated in the supernatants, as previously described for the spectrophotometric measurement of complement-mediated hemoglobin release.

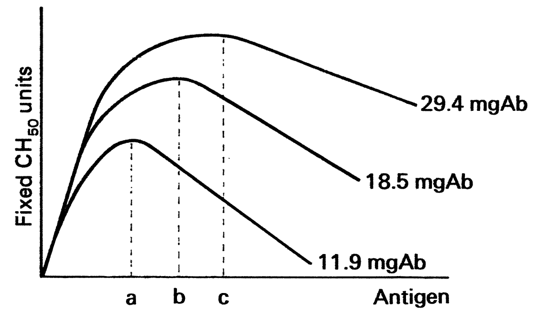


Fig. 7.15. Quantitative complement fixation studied by the macromethod of Mayer et al. in systems with constant levels of antibody (11.9 mg, 18.5 mg, and 29.4 mg) and variable amounts of antigen

Quantitative Testing Methods. Two methods for quantitative testing should be mentioned here:

1) In the *macromethod of Mayer and associates*, dilutions of serum (or of antigen) are incubated for 20 h at 2°–4 °C with an optimum dose of antigen (or of serum) and an excess of complement, e.g., 100 CH_{50} units. Controls containing just serum + C or antigen + C are also included in the test, in order to detect any anticomplement activity in the reagents. After incubation the mixtures are diluted to measure the quantity of unfixed C and to determine the number of fixed CH_{50} units.

2) In *serodiagnostic tests* in which small quantities of complement (2–5 CH_{50} units) are used in such a way that the residual quantity of unfixed C is of the order of 0.8–1.2 CH_{50} units, the amount of complement fixation can be determined directly by addition of EA to the undiluted mixtures. Included in this category are the quantitative techniques (perhaps better described as semiquantitative) of Christiansen, of Maltaner and associates, of Stein and Van Ngu, and of others.

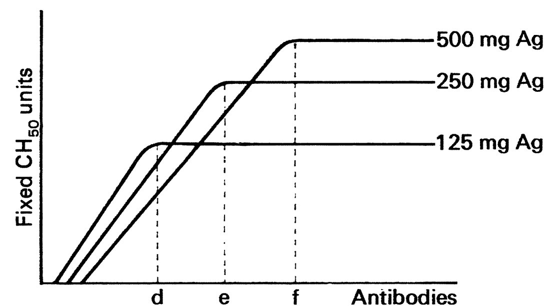


Fig. 7.16. Quantitative fixation of complement studied by the macromethod of Mayer et al. in systems with constant amounts of antigen (125 mg, 250 mg, and 500 mg) and variable amounts of antibody

The introduction of Mayer's method permitted investigators to establish with precision the relations between antigen and antibody in the complement fixation reaction. By maintaining the antibody dose constant and varying the concentration of Ag, a CF curve is produced that notably resembles the curve of specific precipitation, clearly exhibiting a zone of inhibition by excess antigen (Fig. 7.15). However, when a fixed dose of Ag is added to a varying dose of immune serum, the curve changes and does not exhibit a zone of inhibition (Fig. 7.16). Still, in both cases the quantity of fixed C reaches a maximum that corresponds to the maximally reactive dose of antigen (in the tests with constant antiserum) or of serum (in the tests with constant antigen).

If we represent graphically the quantities of serum on the abscissa, and the number of units of complement fixed in the presence of maximally reactive doses of antigen on the ordinate, a sigmoid curve is obtained (Fig. 7.17). The fixation potency, expressed in terms of CF_{50} (being the dose of serum at which 50% of the complement units involved are fixed) is found at the linear portion of the curve.

In the semiquantitative techniques, the maximally reactive dose of antigen is not established for each dilution of antiserum;

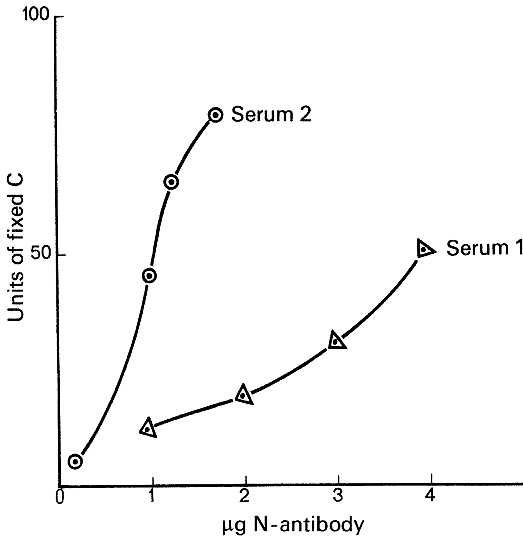


Fig. 7.17. Sigmoidal curve of the number of units of complement fixed by varying quantities of antibody, in the presence of optimum amounts of antigen. Determination of CH_{50}

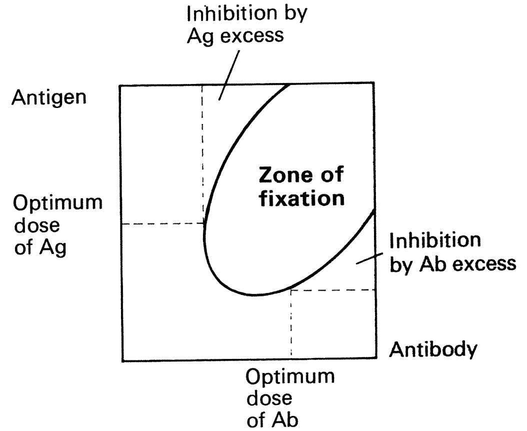


Fig. 7.18. Isofixation curve for the determination of the optimum concentration of antigen

rather, a dose capable of reacting optimally with a series of serum dilutions is utilized. To establish this dose, it is necessary to determine the curve of isofixation through experiments of the "checkerboard" type, in which the antigen and the serum are varied in perpendicular directions. The optimum doses are indicated by the minimum quantities of antibody (or antigen) and can be visualized easily by inspection of the isofixation curve (Table 7.2 and Fig. 7.18).

Under the conditions of the semiquantitative test, Maltaner and his associates verified the direct proportionality between *K'sa, the number of units of C required for 50% hemolysis in the presence of serum plus antigen,* and $1/D$, *the inverse of the serum dilution,* which permitted expression of the fixative titer in terms of the angular coefficient (slope) of the line $K'sa = b'(1/D)$. Since $K'sa$ is calculated by dividing n, the number of units of C used in the test, by the correlation factor (f) corresponding to the percentage of lysis, then

$$b' = D \times (n/f), \tag{1}$$

in which $f = (y/1-y)^h$ and h is the slope verified for the titration of the complement in the presence of serum or of antigen alone.

Table 7.2. Semiquantitative determination of the optimum antigen dose in the bovine serum albumin-rabbit antibovine serum albumin system in a test with five units of complement

Antibody µg N/ml	Antigen µg N/ml							
	0.001	0.012	0.04	0.12	0.36	1.10	3.33	1.00
3.3	1[a]	0	0	0	0	0	0	0
2.2	2	0	0	0	0	0	1	1
1.5	4	0	0	0	0	0	1	4
1.0	4	1	0	0	0	1	4	4
0.66	4	3	1	1	1	4	4	4
0.44	4	4	2	3	4	4	4	4
0.30	4	4	4	4	4	4	4	4

[a] 0, 1, 2, 3, and 4 represent, respectively, 0, 25%, 50%, 75%, and 100% hemolysis

More precise experiments have shown, however, that the rigorously linear relation is that which is observed between $\log D$ and $\log(y/1-y)$, in accordance with the equation $\log D = \log T + h's \times \log y/1-$ in which $h's$ is the angular coefficient corresponding to the quantity of residual complement after the fixation reaction. From this equation, the following formula results for the calculation of fixative titer:

$$T = D \times (1/f') . \qquad (2)$$

Obviously, if $h = h's$, formula (1) gives a value equal to that of formula (2) or a multiple thereof. For example, if a serum at a dilution of 1/25 in the presence of the optimum antigen dose and in a test with 6 units of complement, produces 75.5% hemolysis where h is equal to $h's = 0.2$, and $f' = f = 1.25$, then the values of the titers calculated with the two formulas are:

$$b' = 25 \times 6/1.25 = 120,$$

$$T = 25 \times 1/1.25 = 20.$$

However, depending upon the number of units of C utilized in the test, the nature of the antigen or serum, and other factors, the value of $h's$ can differ considerably from that of h, imparting a corresponding difference to the calculation of fixative titer. In these cases, to avoid the calculation of new conversion factors, the titer can be determined graphically, as indicated in Fig. 7.19.

Mechanism of Complement Fixation. The mechanism of the fixation of complement is still obscure. Even the early immunologists sought to interpret it, with Ehrlich maintaining that complement fixation operates at the level of a special group (a complementophil group) of antibody binders (amboceptors), whereas Bordet attributed the phenomenon to the absorption properties of the antigen–antibody complex.

Modern immunologists, influenced by the works of Ishizaka and others, tend to favor the original point of view of Ehrlich, attributing primarily to the antibody the capacity to fix complement: (1) The immunoglobulins capable of fixing C in the presence of antigen also do so when aggregated by nonspecific means, such as by heat and by bisdiazobenzidine. (2) The binding property resides in the Fc fragment of the antibody molecule, because only this fragment becomes anticomplementary when aggregated, unlike the $F(ab')_2$ fragment. (3) When two immunoglobulins react, one an antigen and the other an antibody, C binding is observed only when the antibody immunoglobulin is capable of fixation – e.g., CF positive with rabbit anti-fowl gamma-globulin antibody, but not with bird anti-rabbit gamma-globulin, if rabbit or guinea pig serum is used as complement.

Relevant experimental data indicate, however, that simple aggregation is not sufficient to activate complement, and suggest that, in combining with the antigen, the antibody molecule exposes certain structures previously occluded in the C_H2 region of the

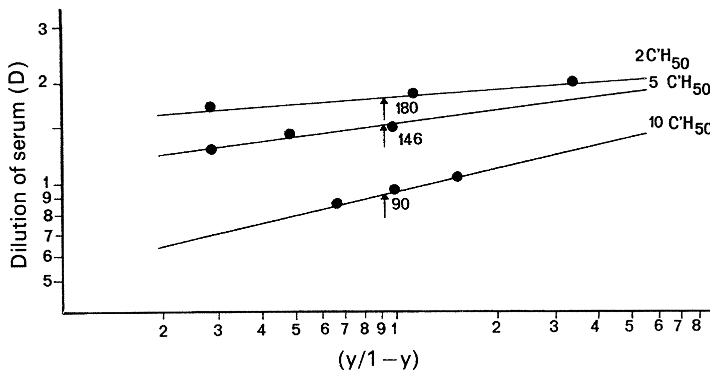


Fig. 7.19. Graphic determination of the fixation titer in the function of the curve log D versus log $(y/1-y)$

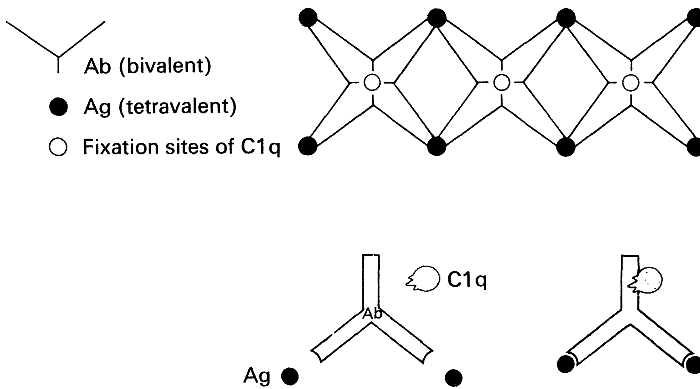


Fig. 7.20. Mechanism of complement fixation by the antibody after its interaction with the specific antigen

Fc fragment, by a conformational mechanism comparable to the allosteric modification of enzyme molecules (Fig. 7.20).

In any case, electron microscopy shows that fixative capacity is associated with the formation of aggregates of four or more molecules of complete antibody, but not of Fab fragments (Fig. 7.21).

Effects of Complement Fixation on Cell Membranes. The fixation of components of complement to the surfaces of cells gives rise to a series of manifestations that can be catalogued as follows:

1. Effects arising from fixation of C1 – C9
   (a) Immunocytolysis
   (b) Immunocytotoxicity
2. Effects arising from fixation of C1 – C3
   (a) Immunoadherence
   (b) Immunoconglutination.

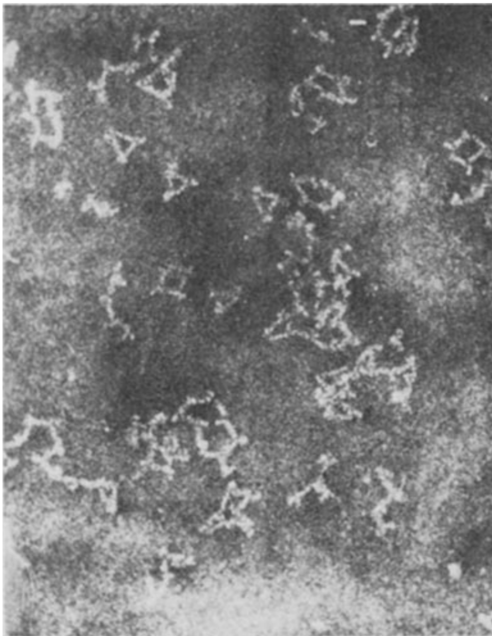


Fig. 7.21. Electron micrograph of antibody–hapten aggregates endowed with maximum capacity for complement fixation [Partial reproduction from Valentine RC, Green NN (1967) J Mol Biol 27:615

Immunocytolysis

Even in the early days of immunology, it was observed that bacteria or red blood cells, "sensitized" by the specific antibody, were lysed upon the addition of complement. We have already referred in detail to the mechanism of specific hemolysis, i.e., the sequential action of the C1–C9 components of complement. In this section, we deal only with the phenomenon of specific bacteriolysis, first observed in vitro after the inoculation of cholera vibrios into the peritonium of immunized rabbits. Under these conditions, whereas in the control animals examination of the peritoneal exudate revealed the presence of *V. cholerae* with its typical morphology and mobility, in immunized rabbits the vibrios quickly lost their mobility and disintegrated into granules (Pfeiffer's phenomenon). The phenomenon can be studied conveniently in vitro by mixing bacteria, immune serum, and complement, and determining the number of viable bacteria (bactericidal effect) by distributing the suspension on an agar plate and counting the colonies that are formed. Generally speaking, we

can say that gram-negative bacteria (e.g., *V. cholerae*, *S. typhosa*, *S. dysenteriae*, *E. coli*, *P. aeruginosa*) are lysed and destroyed, whereas the gram-positive (e.g., gram-positive cocci, *B. subtilis*) are inhibited in their growth without concomitant lysis. In both cases, however, the cytocidal effect depends upon the sequential action of the nine components of complement: Serum of rabbits deficient in C 6, for example, is incapable of exercising bactericidal action on *S. typhosa*.

With red blood cells, lysis appears to depend exclusively upon the initial formation of ultramicroscopic lesions on the cell membrane, with an initial increase in the permeability to substances of low molecular weight (entry of H_2O and Na, exit of K), followed by distention and rupture of the membrane and by permeability to substances of high molecular weight (e.g., hemoglobin). In bacteria, however, this condition is not sufficient, because alteration of the cell wall is also necessary.

In gram-negative bacteria rich in phospholipids and with thin cell walls (10 mμ or less), the combining action of antibody and complement leads to the formation of damaged and defenseless spheroplasts, susceptible to lysis; in gram-positive bacteria, however, that have thick cell walls (15–50 mμ) and are poor in lipids, conditions are not favorable to the disintegration of the cell walls and lysis does not occur, although the lesion of the cytoplasmic membrane can have a bactericidal effect. Two experimental facts support this interpretation: (1) *E. coli* spheroplasts and *B. subtilis* protoplasts are lysed by the action of specific antibody plus complement; and (2) bacteria resistant to the action of antibody plus complement undergo lysis when lysozyme is added, which by destroying the cell wall exposes the damaged protoplasts.

The lysozyme is probably not the only nonspecific adjuvant factor that operates in specific bacteriolysis. It is possible that the bactericidal actions of normal serum, though generally attributed to natural polyspecific antibodies, are actually caused by serum factors nonspecific for the effect produced by small levels of specific complement-binding and complement-activating antibodies. Also not to be excluded is the hypothesis relating to the activation of complement by serum factors not related to antibodies – or even as a result of properties of the bacterial surface itself.

It is pertinent to mention the serum factor called properdin (from Latin perdere "to destroy"), which was described originally as a protein capable of combining with constituents of the cell walls of different microorganisms (including the mixture of polysaccharides of the yeast cell wall, called zymosan). It also was thought to have a bactericidal effect in the presence of complement and Mg. Apparently not an antibody, it is like an antigen–antibody complex that causes the elective fixation of C 3, consuming practically no C 1, C 4, or C 2.

The existence of properdin has been confirmed by biochemical experiments utilizing chromatographic fractionation to separate a β-globulin with a molecular weight of 223,000 daltons, homogeneous under electrophoresis and ultracentrifugation, and incapable of reacting with antibodies against IgG, IgA, IgM, or their heavy and light chains. This protein exhibits reactions characteristic of properdin – in particular, the capacity to inactivate C 3 through prior combination with a serum proactivator (C 3 PA). Its operative mechanism therefore approximates that of endotoxins and that of the so-called thermolabile opsonin of pneumococcus, which activates C 3 by an alternative means independent of C 4 b 2 a.

The betalysins may also be mentioned among the nonspecific bactericidins of normal serum; their action, independent of complement, operates predominantly on gram-positive bacteria. Such substances are liberated only after the coagulation of the blood, and do not appear to exercise any important function in vivo.

Immunocytotoxicity

Immunocytoxicity describes antigen–antibody–complement interaction with the surface of a cell that does not result in cytolysis,

but is accompanied by cytotoxicity activity, or structural alterations, and disturbances of cellular function (immobilization, increase in permeability, metabolic alterations).

The immobilization of *T. pallidum* (TPI test) by sera of patients with syphilis is an example of a reaction of this type. Mobile treponemas are mixed with the patient's serum and guinea pig complement. If the reaction is positive after incubation at 37 °C for 16–18 h, there is immobilization of the treponemas; there is no immobilization when normal serum is added or when complement is omitted. The long incubation time is necessary because the treponemas possess a coating of hyaluronic acid that impedes access of the antibody to the antigenic determinants (proteins) of the spirochete. The addition of lysozyme hastens the reaction.

An example of a cytotoxicity test that involves an increase in cellular permeability is the lymphocytotoxicity test, used currently to disclose histocompatibility antigens, with a view to selecting donors for tissue or organ grafts. A purified suspension of human lymphocytes is mixed with antiserum and complement (human, rabbit serum). After incubation at 37 °C, trypan blue (or eosin) is added. Under the microscope it can be seen that the injured cells have taken up the stain and appear blue (or dark red), whereas intact cell membranes do not allow the uptake of stain (microdye-exclusion test).

Experimental glomerulonephritis, induced by the inoculation of heterologous antikidney serum (Masugi's nephritis), furnishes an interesting example of direct immunocytotoxicity (reaction with antigens of the glomeruli) and indirect immunocytotoxicity (reaction with heterologous antigens fixed to the glomeruli). If rabbit anti-rat kidney serum is injected into a rat, intense, precocious proteinuria occurs (when the antiserum dose is sufficient), resulting from the cytotoxic action of the anti-kidney antibody upon antigens belonging to the basement membrane (BM) of the glomeruli. Upon ultramicroscopic examination, a uniform thickening of the BM may be clearly ascertained, and immunofluorescence discloses the presence of rabbit immunoglobulin and thickening of the capillary lumen (mesangial pattern) along the endothelial face of this membrane. The role of complement in nephrotoxic nephritis is clearly indicated (1) by the absence of glomerular lesions in complement-depleted animals; and (2) by the incapacity of the pepsin fragment of the nephrotoxic antibody to produce immediate proteinuria caused by the complete complement-fixing antibody. If, however, a non-complement-fixing antibody, is injected instead of a complement-fixing antibody, the proteinuria does not develop immediately. This process occurs in the classic experiment in which goose anti-rabbit kidney serum is injected into a rabbit. Since the antibody is incapable of fixing complement, proteinuria appears only after the heterologous immunoglobulin is bound to the BM and subsequently provokes the formation of rabbit anti-goose immunoglobulin, which then binds complement and is responsible for the delayed proteinuria (indirect immunocytotoxicity).

Distinct from nephrotoxic nephritis, the nephritis produced by the deposition of irregular masses (lumpy-bumpy pattern) of antigen–antibody–complement complexes is readily disclosed by immunofluorescence of the reactants involved.

Another example of indirect immunocytotoxicity is provided by thrombocytopenic purpura, which develops in certain cases of allergy to drugs, e.g., in cases of hypersensitivity to allylisopropylacetylurea (Sedormid) or to quinidine. In these cases, the specific antibody reacts with the drug, which is fixed to the platelets; complement is activated through the antigen–antibody complex, resulting in thrombocytolysis and consequently thrombocytopenia. Lysis of platelets can be achieved in vitro by mixing the patient's serum with normal platelets and the drug, along with complement. The serum of the sensitized individual causes lysis of the platelets; lysis does not occur in control tubes containing normal serum to

which the drug or complement has not been added.

Immunoadherence

Certain microorganisms, such as spirochetes and trypanosomes, when mixed with the specific antibody in a suspension of platelets and in the presence of complement, adhere to the platelets forming grains clearly evident by dark-field microscopic examination (Rieckenberg reaction). This phenomenon has been reinvestigated, and today it is called immunoadherence (IA) – the adhesion of antigen–antibody–complement complexes to the surfaces of the erythrocytes of primates[6] or to the platelets of other species, microscopically and macroscopically evidenced by the agglutination of the indicator particles.

Analysis of the role of complement in IA has shown that only the C 1 and C 3 components are involved, with the critical part being played by fixed C 3; equal immunoadherence capacity is exhibited by the EAC $\overline{1,4,2,3}$; EAC $\overline{1,4,3}$; or EAC $\overline{4,3}$ complexes. Immunoadherence is an extremely sensitive serologic reaction that can be used for detecting minimal quantities of autoantibodies (e.g., in detecting levels of autoantibodies not disclosed by other reactions), or for the titration of C 3. In addition to its serologic value for C 3 titration and as an indicator for the complement binding reaction, an important role is attributed to immunoadherence in phagocytosis: Erythrocytes or bacteria, treated with specific antibodies, can bind complement, and the coated C 3 b particles adhere not only to erythrocytes but also to the receptors of the macrophages.

Immunocytoadherence. Biozzi and associates used this term to describe rosettes formed by erythrocytes around the lymphocytes of immunized animals (Fig. 7.22). Thus, for example, the lymphocytes of the spleens of normal mice are capable only in small numbers of forming rosettes with sheep erythrocytes. If, however, mice are immunized with these erythrocytes, the number of rosettes increases gradually from the fourth day and in amounts paralleling the agglutination titer of the serum. The phenomenon is interpreted in terms of the formation of antibodies at the cellular level prior to their liberation by the lymphocytes that produced them. Interesting data have been obtained with this technique concerning the cytodynamics of the formation of antibodies.

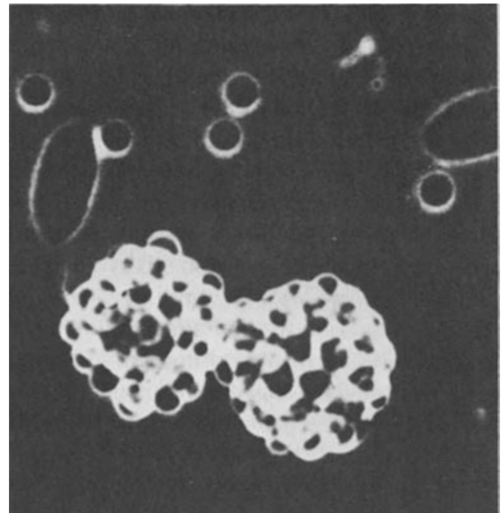


Fig. 7.22. Immunocytoadherence (Original of B. Biozzi)

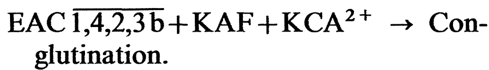
Conglutination and Immunoconglutination

Conglutination is the active agglutination by a euglobulin[7] existing in bovine serum called conglutinin (K) of sensitized erythrocytes (E′A) that are coated with complement (C). Fresh horse serum is generally

6 In the case of human erythrocytes, the receptor is destroyed by neuraminidase

7 The conglutinins can be purified through adsorption by zymosan in the presence of Ca^{2+} and subsequent elution by EDTA. The result is a highly asymmetric molecule, 7.8 S, mol. wt. 750,000, not related to the gamma globulins, with an elevated level of glycine (18%). It is resistant to heating to 56 °C and to treatment with ammonium salts, mercaptoethanol, neuraminidase, and pepsin; however, it is easily destroyed by trypsin and by papain

used in the conglutination reaction in a dose sufficient to provide an adequate quantity of C1, C4, C2, and C3, in the absence of hemolysis. The critical component is C3, which, when fixed (C3b), is under the influence of a serum β-globulin (mol. wt. 100,000), probably an enzyme: this is the conglutinogen-activating factor (KAF); which exposes the polysaccharide determinants, which in turn combine with K in the presence of Ca^{2+}, producing the conglutination phenomenon



$$EAC\,\overline{1,4,2,3b} + KAF + KCA^{2+} \rightarrow \text{Conglutination.}$$

The conglutination reaction is important in serodiagnostics, since, like the specific hemolysis reaction, it serves as an indicator of free C in complement fixation tests (e.g., in the serodiagnosis of glanders).

Immunoconglutination is the agglutination of $EAC\,\overline{1,4,2,3b}$ by anti-non-gamma-autoantibodies of a specificity directed against determinants exposed on fixed C4 and C3. Such autoantibodies, called immunoconglutinins (IK), can be produced experimentally by the injection of bacteria that are sensitized (or else in vitro by a heteroantibody) and coated with complement (heterostimulated IK); they also are produced naturally, during infections, by microorganisms that coat the antibody and autologous complement (autostimulated IK).

A role in nonspecific resistance to infection has been attributed to the immunoconglutinins, which through an opsonizing process promote phagocytosis and intracellular digestion of bacteria by macrophages of the reticuloendothelial system.

Serologic Reactions in Vivo

Phagocytosis and Opsonization

Live cells have the capacity to engulf particles through an active process that involves the formation of hyaloplasmic membranes and bears the general term endocytosis: The term phagocytosis is used specifically for solid particles (from Greek *phag*, as in *phagein*, "to eat"), and pinocytosis is used with regard to the incorporation of liquids and the particles dissolved in them (from Greek *pin*, as in *pinein*, "to drink").

Whereas phagocytosis is characteristic of cells called phagocytes, pinocytosis is exhibited by all cells and probably constitutes a particular case of phagocytosis of inframicroscopic particles and macromolecules.

In both cases, the mechanism of engulfment is fundamentally identical. It is initiated by the adhesion of a particle to the cytoplasmic membrane, followed by invagination, which deepens little by little and ends by sequestration of the particle in a cytoplasmic vacuole. At the same time, re-formation of the cytoplasmic membrane proceeds at the point of invagination (Fig. 7.23). It was formerly believed that phagocytosis depended upon a purely physical mechanism, i.e., the interplay of the forces of surface tension. However, today it is known that this mechanism requires supplementary energy through relevant modifications of the metabolism of the phagocyte, represented by a conspicuous increase in anaerobic glycolysis of the polymorphonuclear leukocytes (see Chap. 11).

Phagocytosis, which in lower organisms represents the essential mechanism of nutrition (intracellular digestion), is also of fundamental importance for cleansing the organism (by scavenger cells) through the removal of waste products of internal origin

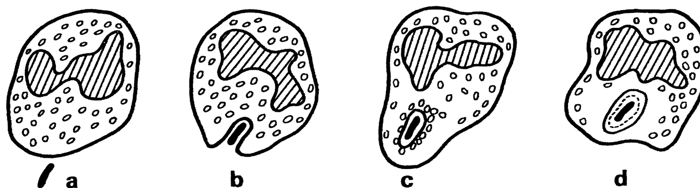


Fig. 7.23 a–d. Successive phases of phagocytosis. **a** Before engulfment, **b** cytoplasmic invagination, **c** formation of the phagocytic vacuole, **d** degranulation: evacuation of the enzymatic content of the lysosymes into the phagocytic vacuole

(e.g., dead cells or components of injured cells, denatured macromolecules), or through the elimination of foreign bodies, including microorganisms, regardless of their nature. As ingeniously recognized by Metchnikoff at the end of the last century, this latter process constitutes the basic defense mechanism against infections – in lower organisms as well as in the higher animals. In the latter, the digestive function becomes extracellular by means of enzymes situated in the gastrointestinal tract; however, some cells of mesenchymal origin still endure, scattered throughout the organism (fixed cells of the reticuloendothelial system) or accumulated at the sites of local inflammation, constituting an effective barrier to the penetration and dissemination of infectious agents, especially in immunized animals.

The Phagocytic Cells. In vertebrates, Metchnikoff distinguished two classes of phagocytes – microphages and macrophages.
Microphages correspond to polymorphonuclear leukocytes of the blood, capable of phagocytosis (neutrophils and eosinophils), whereas macrophages are cells found throughout the organism and include (1) the monocytes of the blood; (2) the endothelial cells of the hepatic (Kupfer cells), splenic (red pulp), and lymphatic sinusoids; and (3) free phagocytes encountered in the tissues (e.g., in the milky spots of the omentum) and in inflammatory exudates (e.g., in peritoneal exudate and in pulmonary alveoli).
As vital staining methods advanced, it became possible to characterize the macrophage system better on the basis of a common physiologic property. This property is granulopexis, or the capacity of macrophages to capture electronegative-staining colloidal micelles (trypan blue, lithium-carmine, etc), or micelles of colloidal carbon, accumulating them in the form of granules in their cytoplasm.
For the group of cells endowed with the granulopexic function, Aschoff proposed the name reticuloendothelial system (RES). This system includes:
1) The monocytes of the blood
2) The histiocytes of the tissues
3) the microglia of the central nervous system
4) The reticular cells (weakly active) of the lymphatic tissues
5) The endothelial cells (very active) that coat the lymphatic and blood sinusoids (liver, spleen, bone marrow, adrenals, and anterior pituitary).

The macrophages encountered in the inflammatory exudates are thought to originate from monocytes in the blood or from tissue histiocytes.

Quantitative Study of Phagocytosis of Inert Particles by the RES. It is possible to study quantitatively the phagocytosis of inert particles by the RES by injecting into the vein of an animal a known quantity of a suspension of uniform-sized particles, sufficiently large that they cannot be eliminated by the blood. Under these conditions, study of the elimination of the inoculated colloid with time (elimination curve) permits evaluation of the intensity of the phagocytic function exercised by the reticuloendothelial cells that enter into contact with the blood. The quantitative relationship between the concentration C, at a particular time t, and the initial concentration C_0 is expressed by the equation

$$C = C_0 \times 10^{-kt}.$$

From the preceding equation, the value of k can easily be calculated (the phagocytic or granulopexic index) by

$$k = \frac{(\log C_0 - \log C)}{t}, \text{ or } \frac{(\log C_1 - \log C_2)}{(t_2 - t_1)},$$

which measures the phagocytic efficiency of reticuloendothelial cells that enter into contact with the injected colloid (Fig. 7.24).
The value of k is inversely proportional to the quantity of colloid injected (d), so that the product kd is essentially constant for each animal species. Thus, the kd product in

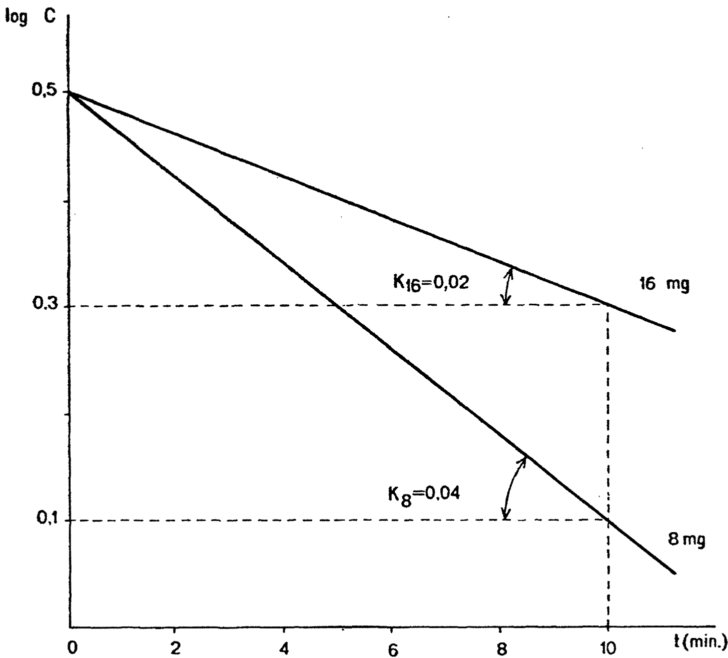


Fig. 7.24. Determination of the phagocytic index based upon the linear regression between the logarithm of the concentration of circulating carbon and the time elapsed

the rat equals 0.208 in tests performed with colloidal carbon, which is to say that for a dose of carbon equaling 8 mg/100 g, the value of k equals 0.208/8 or 0.026, and that for a double dose, k is two times smaller (0.013).

In repeated tests, the values of k for constant doses of colloid in the same animal species vary considerably, e.g., in rats injected with 8 mg/100 g of colloidal carbon, $k = 0.026 \pm 0.015$. This variation is not due to a corresponding variation in phagocytic activity, but to a variation in the weight of the active tissue, i.e., the hepatosplenic tissue (W_{fb}), in relation to the total weight of the animal (W_t). In numerous determinations, it was verified that the k values were inversely proportional to the cube of the W_t/W_{fb} ratio; thus, multiplying the ratio by the cube root of k, a new constant is obtained – α (corrected phagocytic index), which expresses the phagocytic activity as a function of the relative weight of the active tissue:[8]

$$(W_t/W_{fb})^3 \times K = \alpha^3,$$
$$\alpha = (W_t/W_{fb}) \times \sqrt[3]{k}$$

Unlike k, the α index is subject only to small variations ($\pm 10\%$), and it is effectively constant for any animal species, as illustrated by the 8 mg/100 g dose:

Index	Rat	Mouse	Rabbit
k	0.026	0.047	0.08
α	5.4	5.4	6.0

Quantitative Study of Phagocytosis of Bacteria by the RES. Applying the preceding method to the study of the elimination from the blood of bacteria labeled with radioactive isotopes, the identical quantitative relationship was confirmed. However, contrary to what occurred with the inert particles, the dose involved was not found to affect the value of the phagocytic index. This is easily understood when one considers that the

8 The active tissue is practically all represented by the hepatosplenic mass: After injection of colloidal carbon into mice, 90% can be recovered from the liver, 4% from the spleen

phagocytosis of bacteria (and not that of inert colloids) depends upon their interaction with serum components called opsonins, and therefore is subject to the influence of this limiting factor. Under these conditions, above a certain critical value of inoculum, the number of microorganisms phagocytosed corresponds to the maximum number that interacted with the limited level of opsonins available in the plasma of the animal involved.

Normal Opsonins and Immune Opsonins. It has long been known that the phagocytosis of microbes is facilitated by certain proteins existing in normal serum and, in a much more accentuated fashion, by specific antibodies against antigenic determinants of the microbial surfaces.

These substances are called opsonins (Greek *opsonein* "to soften food"), and the process is called opsonization; those in normal serum are called normal opsonins, whereas those in specific antisera are called immune opsonins.

Classically, immune opsonins (formerly called bacteriotropins) were distinguished from natural opsonins by their thermostability; the former remain active after being heated to 56 °C, whereas the latter are destroyed. This thermolability is explained by the fact that the normal opsonins require complement in order to be effective, whereas opsonization by specific antibodies, though it is enhanced by complement, is clearly evident in the absence of complement.

It is still not possible to define with precision the facilitating factors in normal serum phagocytosis; but we can relate them to the activation of the C 3 and C 5 components of complement through the mediation of natural antibodies in low titers or through a thermolabile serum factor (heat-labile factor or HLF) of a non-gamma-globulin nature.

The Role of Complement in Phagocytosis and the Mechanism of Ingestion. The action of complement in fostering phagocytosis can be evidenced clearly by in vitro and in vivo experiments. In the former, through study of the phagocytosis of sensitized erythrocytes (EA) treated with the various complement components, it has been verified that the phagocytosable complex corresponds to EAC 1,4,2,3, in which the effect of C 5 is minimal. Nevertheless, experiments with encapsulated pneumococci treated with fresh guinea pig serum evidenced participation, albeit to a lesser degree, of the C 5 component. With in vivo experiments, the role of complement in phagocytosis is clearly indicated by the retarded elimination of labeled bacteria from the serum of animals lacking complement.

The relevant role of C 3 can be interpreted as a function of the confirmed existence of receptors for C $\overline{3b}$ on the surfaces of polymorphonuclear cells and of monocytes, thereby assuring the initial stage of phagocytosis, i.e., the adhesion of the opsonized particle to the surface of the phagocyte.

The engulfment itself utilizes high affinity receptors for Fc on phagocytes. Following opsonic adherence through C 3 b, the Fc portion of the opsonizing IgG antibody moves along the Fc receptors acting like a zipper to sequester the particle in a phagocytic vacuole (phagosome), which in a later stage becomes a phagolysosome. The mechanism described above corresponds to the classical opsonization of humoral immunity and involves mainly polymorphonuclear leukocytes. Monocytes may also participate in this process, since they are equally provided with C 3 b and Fc receptors. However, the ingestion by macrophages is rather more associated with cellular immunity through the interaction with immune T lymphocytes, as described in a subsequent section (see Immune macrophages). Macrophages may also be armed with cytophilic antibodies or similar factors, e.g., the "specific macrophage arming factor" (SMAF) and thus be enabled to ingest the particle with or without previous opsonic adherence.

Determining Opsonin Concentrations. The classic methods for finding opsonin concentrations consist of comparing the average number (N') of bacteria phagocytized in a

mixture of bacteria and leukocytes suspended in a medium containing the serum under study, with the value N, obtained with an identical mixture suspended in normal serum. The N'/N quotient represents the opsonization index of the serum in question. Thus, for instance, if in a counting of 50 leukocytes we encounter 120 phagocytized microorganisms in the suspension containing the patient's serum, and 200 in the suspension containing normal serum, we may conclude that the opsonization index of the patient's serum is 120/200 or 0.6, i.e., that the serum of the patient possesses 60% of normal opsonization power. A variant of this technique is the opsonocytophagic test, formerly used in the serodiagnosis of brucellosis. Today such tests have fallen into disuse, for they furnish no more diagnostic information than the simple direct or passive agglutination test.

For quantitative determination of the levels of opsonins in sera, the best method available is that based on the rapidity of elimination from the blood of bacteria labeled with radioactive isotopes. To avoid variations resulting from the level of natural opsonins in the experimental animals, a suspension of bacteria is injected and the initial k value is determined in the absence of the immune serum. Then the serum to be studied is injected and the new value of the phagocytic index, k', is determined. The difference, $k'-k$, permits calculation of the number of opsonizing units of the serum in question (Fig. 7.25). If we arbitrarily designate 0.01 as the value $k'-k$ for one opsonizing unit, we can evaluate the number of opsonizing units (OU) of any serum using the formula

$$\mathrm{OU/ml} = 1/\mathrm{V} \times \mathrm{D} \times 100\ (k'-k)\,,$$

in which V and D represent, respectively, the volume and the inverse of the dilution of the serum injected. Thus, for example, if in the testing of a mouse we obtain a k value of 0.01 and k' equals 0.05, for the injection of 0.1 ml of the 1:500 dilution of a determined antiserum, we can conclude that the level of

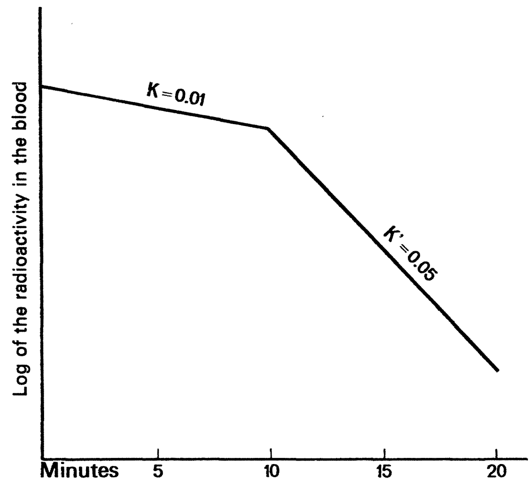


Fig. 7.25. Determination of immune opsonins by the velocity of elimination of *S. typhosa* labeled with ^{131}I. Biozzi et al., 1963

opsonizing units is equal to

$$1/0.1 \times 500 \times 100\ (k'-k),$$
or 20,000 OU/ml.

The application of this method has indicated an impressive correlation between the specificity of the agglutination and that of the opsonizing activity in anti-salmonella sera. For example, different O variants of *S. typhimurium* (4,12; 1,4,12; 4,5,12; and 1,4,5,12) could be opsonized by the monospecific antiserum (anti-1,4,5 or 12) only if it possessed the respective antigenic determinants. The anti-H antibodies did not have any opsonizing power.

Postendocytic Phenomena: Intracellular Digestion. After the ingestion of opsonized particles, due to increased anaerobic glycolysis, strong acidity (pH 3–pH 6) develops at the level of the phagocytic vacuoles. Moreover, phase microscopy discloses that degranulation of the polymorphonuclear vesicles takes place around the vacuoles – the granules being lysosomes containing numerous enzymes, in particular, acid and alkaline phosphatases, ribonuclease, deoxyribonuclease, and β-glucuronidase. Electron microscopy reveals, in addition,

that the vacuole membranes fuse with the membranes of the adjacent lysosomes, which empty their enzymatic contents directly into the site in which the phagocytosed bacteria are encountered. This occurs without exposure of the cytoplasm of the phagocyte, which is thus spared from the possible fatal effect of the enzymes.

Aside from the lysosomic enzymes mentioned, two substances must be specially mentioned that are also liberated by lysosomes and probably constitute important agents in intracellular digestion: lysozyme and phagocytin.

Lysozyme is an acetylamino-polysaccharidase which exists in relatively high concentrations in the polymorphonuclear leukocytes and acts synergically with antibody and complement in the lysis of gram-negative bacteria in vitro, possibly acting similarly in the phagocyte.

Phagocytin is a wide-spectrum, labile protein enzyme that acts upon gram-positive and gram-negative bacteria.

Many other bactericidal substances have been extracted from leukocytes (leukin, leukozymes, etc), but their role in postendocytic digestion is doubtful. The current view is that the intracellular bactericidal effect depends, as does the extracellular effect, upon antigen–antibody–complement interaction, and that the enzymes and other substances liberated by degranulation of lysosomes act only synergically.

Immunophagocytes. Phagocytosed bacteria are not always destroyed by intracellular digestion. Microorganisms such as *D. pneumoniae*, *S. pyogenes*, and *K. pneumoniae* perish rapidly (in 15–30 min) after their ingestion by polymorphonuclear leukocytes; other bacteria, e.g., *M. tuberculosis*, *B. abortus*, *L. monocytogenes*, and *S. typhimurium*, are not destroyed following engulfment but remain alive in a state of latency in the interior of the phagocyte for long periods. The prolonged immunity that develops in response to infection by the bacteria mentioned previously or to immunization with the respective live vaccines (BCG vaccine against tuberculosis, live vaccines against bovine brucellosis) can be attributed to the persistence of such centers of latent or chronic infection. The mechanism by which this immunity develops is unknown. To explain the absence of multiplication of bacteria surviving in the interiors of the phagocytes, it was at first thought that the macrophages of the immunized animal transformed into cells specifically capable of destroying the infectious agent (immunomacrophages). Today it is known, however, that the resistance of the phagocytes is induced by the interaction of the antigen with sensitized lymphocytes, probably through the liberation of lymphokines capable of "activating" the macrophages, making them resistant to the intracellular multiplication of the infecting agent.

It should be noted, however, that although such induction results from a specific interaction, its expression is nonspecific: for example, macrophages of tuberculous animals, resistant to *M. tuberculosis*, also demonstrate resistance to unrelated bacteria such as *Brucella*, *Listeria monocytogenes*, and others.

It is also possible to produce in mice considerable resistance to infection by *S. typhimurium* by various treatments that substantially increase the value of the phagocytic index, k – such as via the injection of live BCG, of endotoxin, or of suspensions of killed *Corynebacterium parvum*.

Neutralization of Toxins

The bacterial exotoxins (diphtheria, tetanus, *Cl. perfringens*, botulin, and others) and the animal venoms (ophidic, arachnidic, scorpion, and others) are highly antigenic proteins that induce the formation of antibodies (antitoxins, antivenoms) capable of neutralizing the effects of the corresponding toxins. Discovered in 1890 by Behring and Kitasato, the antitoxins were the first known antibodies, for which relatively precise methods of titration have long been established.

Table 7.3. Units of measurement for the diphtheria toxin

Unit of toxin	Antitoxin added	Mode of administration	Reaction observed
MLD	–	Subcutaneous	Death in 4 days
MRD[a]	–	Intradermal	Minimum erythematous reaction
L_o[b]	1 AU	Subcutaneous	Minimum edematous reaction
L_r	1 AU	Intradermal	Minimum erythematous reaction
L_t	1 AU	Subcutaneous	Death in 4 days
L_f	1 AU	In vitro	Optimal flocculation

[a] The MRD, minimum reactive dose, is about 250–500 times less than the MLD

[b] Examples of experimental values for the doses above in a toxic filtrate: L_o=0.18 ml, L_t=0.21 ml, L_f=0.155 ml

In Vivo Determination of Antitoxin Concentration. The fundamental research in this domain was performed by Ehrlich with the Diphtheria toxin and antitoxin. To determine the neutralizing level of anti-diphtheria serum, Ehrlich initially used as a base that dose of antiserum capable of neutralizing a test dose based upon 100 minimum lethal doses (MLD). This former unit is called antitoxic unit (abbreviated AU); the latter (MLD) he defined as the minimum 100%-lethal dose (death in 4 days) for guinea pigs weighing 250 g.

The instability of the diphtheria toxin and its progressive transformation into atoxic (toxoid) molecules made it impossible to fix a test dose in terms of MLD. Consequently, Ehrlich had to define it in terms of combination units, through the use of a standard antitoxin, which today is obtained from international reference laboratories such as the National Institutes of Health in the United States.

Practically, it has proved more expedient to read a point from a dose greater than L_0, rather than the neutralization point (L_0). Ehrlich adapted as a point of reference the toxin mixture +1 AU, which kills a guinea pig in 4 days; to the quantity of toxin contained in such a mixture, he gave the name "lethal limit" (L_+). Later, Römer adopted another reference point close to L_0, corresponding to the mixture that produces minimum local erythema when 0.2 ml of the mixture is injected intradermally. He called this new reference dose L_r (reaction limit). The different units of measure of diphtheria toxin are described in Table 7.3; and Fig. 7.26 graphically presents the relationship of AU, L_0, L_r, and L_+ to one another.

The assay of an unknown antitoxin involves two successive determinations: (1) standardization of the toxin, or determination of the test dose (L_r or L_+) in the presence of 1 AU furnished by the standard antitoxin; and (2) assay of the antitoxin by mixing succes-

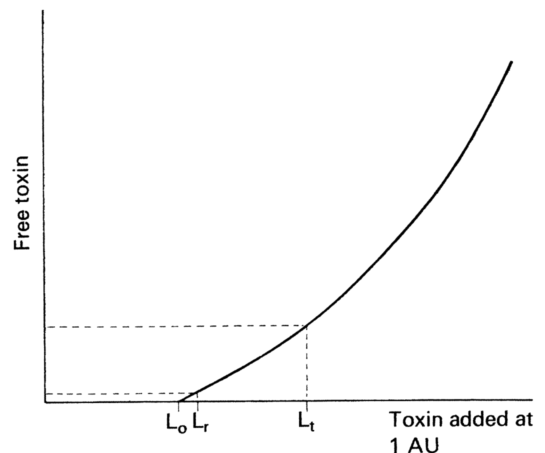


Fig. 7.26. Graphic presentation of the relationship between L_0, L_r, L_t, and AE

sive dilutions of the antiserum containing the previously standardized toxin.

Neutralization of Toxins and Precipitation. When the antitoxins were discovered, precipitation of the toxin–antitoxin (TA) complex was not observed, and the effect of the antibody was characterized only by its in vivo neutralizing power.

In 1922, it was recognized, however, that T and A, in optimum proportions, were capable of precipitation. This caused Ramon to develop an in vitro method for assaying antitoxins.

Quantitative studies of the reaction between T and A yielded curves similar to those obtained with other anti-protein precipitating systems, making it possible to observe a curve of the "horse type" or of the "rabbit type," depending on the origin of the antitoxic immunoglobulin.

In the bell-shaped curve observed in the quantitative study of precipitation of diphtheria toxin by horse antitoxin (Fig. 7.27), there is a maximum point corresponding to neutralization (L_o and L_f in close proximity), and a second point remote from the first that corresponds to the L_t dose.

Immunoglobulin Classes of Horse Antitoxin. Equine antitoxins belong to two subclasses

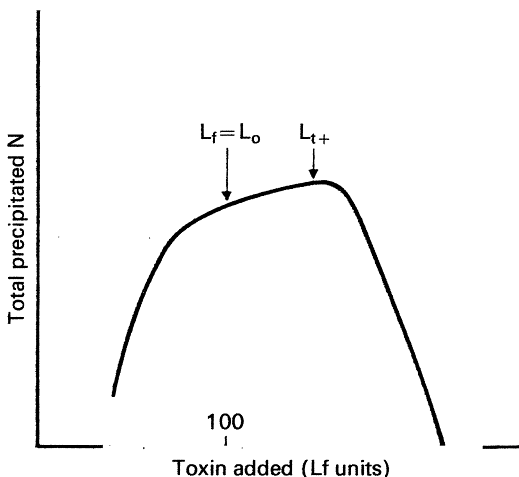


Fig. 7.27. Bell-shaped curve of the precipitation of diphtheria toxin by horse antitoxin, indicating the location of the points corresponding to Lf, L_o, and L_t

of IgG: 1) On initial immunization, to a fast component (γ_2 or γ_1 mobility) termed IgG(T); and 2) on continued immunization, to slow (γ_2) IgG.

The two antibodies can be separated by successive chromatography on DEAE- and CM-cellulose. The γ_2-antitoxin gives the "rabbit type" of precipitin curve (see Fig. 7.30), whereas IgG(T) gives the bell curve characteristic of Ramon flocculating antibodies (Fig. 7.27).

In other species (cattle, sheep, rabbit, monkey, and man) antitoxin is always associated with the slow, IgG fraction.

Avidity of Antitoxins. Certain antitoxins dissociate easily from the TA complex and are called non-avid, in contrast to those that form firm combinations and are thus called avid. This is an important characteristic to be checked for in antitoxic sera, because satisfactory therapeutic results cannot be expected with the use of non-avid antitoxins.

Initially it was observed that certain TA mixtures were innocuous when injected subcutaneously, but demonstrated toxicity upon intravenous inoculation. Later, when it was determined that the toxicity of an injected TA complex was dependent upon its concentration, this effect (no toxicity with subcutaneous, toxicity with intravenous injection) was found to result from the dilution of the TA complex.

Cinader proposed an index for measuring the avidity of antitoxins – the ratio

$$\frac{T/A}{T'/A'}$$

for the quantities of toxin and antitoxin required to constitute neutral mixtures with respect to two levels of toxin, T and T′, with T′ being less than T (for example ½ T). If the Cinader index is greater than 1, the antitoxin is considered non-avid, revealing a capacity to dissociate from the TA complex upon dilution.

Another important value in determining the avidity of antitoxins is the ratio of the in vi-

vo/in vitro concentrations, measured by the quotient

$$\frac{L_t}{L_f} \text{ or } \frac{L_r}{L_f}.$$

Antitoxin flocculates better than it neutralizes and consequently yields L_f values considerably lower than e.g.,

$$\frac{L_t}{L_f}=\frac{0.2}{0.1}=2.$$

With avid antitoxins this ratio generally approaches a value of 1.

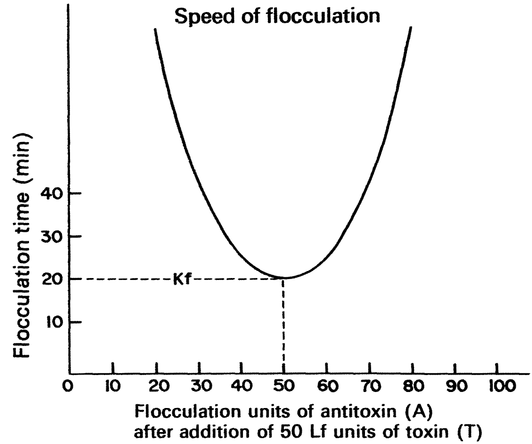


Fig. 7.28. Determination of K_f value

Flocculation. The minimal flocculation time, which corresponds to the complete neutralization of T by A, is called K_f and is inversely proportional to the concentrations of the participating reagents.[9] Thus, if T and A are in concentrated solutions, flocculation occurs rapidly. In fact, in adjacent tubes containing the optimal neutralizing mixture, flocculation occurs almost simultaneously. This prevents an exact determination of the tube corresponding to the flocculation optimum, which again indicates the value of K_f. On the other hand, if T and A are too dilute, only a light, late flocculation occurs, which cannot be clearly defined. Therefore it is necessary to use favorable concentrations, e.g., 2 ml of a 25 L_f/ml solution of toxin and variable amounts of antitoxin with about 50 flocculation units (Fig. 7.28).
Determination of the K_f value is of great practical importance, because it depends not only on the optimal T/A ratio, but also on the inherent properties of the reagents: (1) If a known serum is used, an increased K_f value, i.e., a slow flocculation, suggests an alteration in T. (2) When the same toxin is used, slow flocculation signifies minimal avidity. Purified T and A result in rapid flocculation.

9 The variation of K_f as a function of the concentrations of T and A is expressed as the empirical equation $\log K_f = a - b \log(A+T)$

Mechanism of Neutralization. The mechanism for neutralization of toxins by antitoxins remains obscure but admits of three distinct possibilities (Fig. 7.29): (1) The antitoxin may bind, through competitive inhibition, at the level of the T site responsible for the toxicity. (2) The antitoxin may act at a site near the active T region and hinder the toxic effect by a steric hindrance mechanism. (3) The antitoxin may act upon a distant site and impede toxicity by an allosteric mechanism. With anti-enzymes, which can be considered in the case of antitoxins, possibility (1) can be excluded because for a fixed quantity of antibody, inhibition of enzymatic activity is not influenced by the increase in the concentration of the substrate. It is not possible, however, to decide between hypotheses (2) and (3).
Some experimental support for interpretation (2) is supplied by the fact that total inhibition occurs only when both enzyme and substrate are of low molecular weight, e.g., lecithinase from *Cl. perfringens* (30,000) and lecithin (1,200), with no inhibition occurring when the enzyme is of high molecular weight and the substrate is of low molecular weight, as occurs with the galactosidase (800,000)-lactose (350) system. When the enzyme is small and the substrate is large, e.g., with ribonuclease (14,000) and RNA ($n \times 10^6$), inhibition is partial.

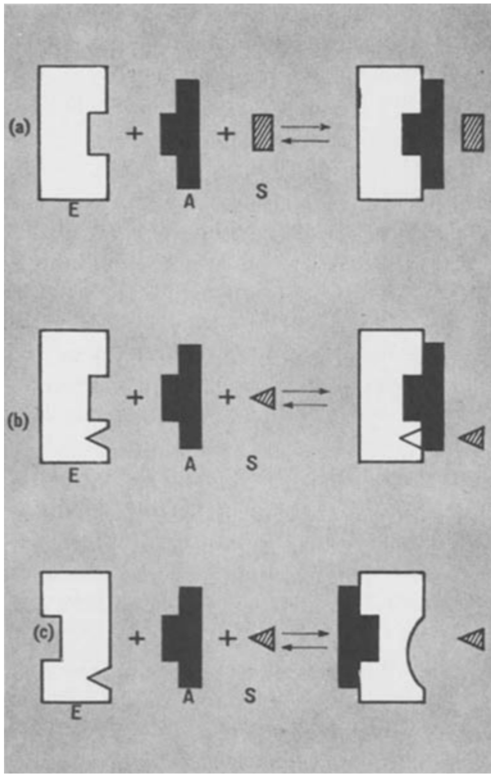


Fig. 7.29. Interpretation of the mechanism by which enzymes are neutralized by anti-enzymes (Original of O.G. Bier)

Protective Action of Antibacterial Sera

The antibacterial sera are capable of passively protecting laboratory animals by the action of opsonizing bactericidal anti-bodies or bacteria directed against surface antigens of the infecting microorganism. For example, anti-pneumococcus serum through its antibodies (carbohydrate S, type-specific) protects mice against pneumococcic infection by an alteration of the capsule, as evidenced by its swelling *(Quellung)*, which fosters the phagocytosis of virulent pneumococci.[10]

Examples of the same type are supplied by anti-*Hemophilus influenzae* serum, which also acts upon the capsular polysaccharide of the microorganism, and by anti-streptococcus serum, whose protective action is due to antibodies against the type-specific M protein of the beta-hemolytic streptococci.

In the case of intestinal bacteria (*V. cholerae*, *S. typhosa*, *S. dysenteriae*, and others), aside from their opsonizing action, antibodies with bactericidal action may play an important part, with or without bacteriolytic effect.

Early immunologists attributed to bacteriolysis (Pheiffer's phenomenon) the role of the fundamental mechanism of immunity, but today it is generally considered that the cytotoxic and cytolytic actions mediated by antibody and complement (C 1–C 9) contribute, overall, as synergic factors within the framework of a more fundamental mechanism represented by phagocytosis followed by intracellular digestion. According to this interpretation, the action of the antibody–complement system resides essentially in a lesion of the cell wall, which conditions the transformation of the gram-negative bacteria into spheroplasts that are either lysed extracellularly, or more frequently, are destroyed in the interiors of macrophages that phagocytose them.[11]

Antibacterial antibodies can be either IgG or IgM, the latter appearing to be more active, whether as opsonizing agents or as bacteriocidins.

Deeply situated antigens that are exposed by the rupture of the capsid, or coat, via the precipitation reaction or fixation by complement, do not appear to be related to the protective action. However, the surface antigens, associated with the sites responsible for the fixation of viral particles to the receptors of the sensitized cells, are considered of vital importance, because they stimulate the production of antibodies capable of impeding the multiplication of the virus and of neutralizing its pathogenic effect.

10 The antibody against somatic carbohydrate C lacks protective action

11 The bactericidal action exhibited by immune sera against *S. typhosa* or *S. typhimurium* notwithstanding, there is evidence that the immunity against these microorganisms is fundamentally cellular and that it lies in their inability to multiply in the interior of the macrophages of an immunized animal (see Chap. 6)

The mechanism for neutralization of viruses is exemplified by the much-studied model of the influenza virus (myxovirus), whose virion contains an internal group-specific S antigen associated with the nucleocapsid of the helical structure and an external V antigen, type-specific, associated with the coat or, more precisely, with the spicula that radiate from the surface of the viral particle. The V antigen is a glycoprotein and corresponds to hemagglutinin, being capable of establishing bridges between the virion and agglutinable erythrocytes. The formation of these bridges is assured by the presence, on the erythrocytic membrane, of a mucoprotein receptor with residual terminals of N-acetylneuraminic acid (sialic acid). Identical receptors are encountered on the surfaces of sensitized cells, and when free in the secretions they act as inhibitors.

In the presence of the anti-V antibody, the hemagglutinin is coated, and hemagglutination is inhibited. The hemagglutination-inhibition reaction can be considered the equivalent in vitro of the neutralization of the pathogenic effect in vivo, attributable to a blocking action of the antibody in relation to the fixation of the virus to the sensitized cells (target cells).

In practice, the in vivo neutralization test is performed by inoculation of a series of dilutions of serum with a constant number of viruses in tissue culture into a fertilized egg or into a sensitized animal, with 50% being adopted as a point of reference for the reading of the results. As with the T–A complex, the virus–antibody complex is reversible, dissociating upon dilution.

The anti-virus antibodies present in the immune sera can be either IgG or IgM immunoglobulins; in the secretions the predominant type is IgA, which is produced locally. Some authors contend that IgA antibodies play a part in immunization against influenza, which was practiced with success in the Soviet Union through nasal administration of attenuated virus. It is also possible that the high degree of immunity obtained in the oral vaccination against poliomyelitis with attenuated virus may be attributed to local production of antibodies capable of neutralizing the virus in the intestines before it reaches the blood.

Quantitative Study of the Antigen–Antibody Reaction

Quantitative Precipitation

Precipitation Curve. Quantitative study of the specific precipitation reaction, initiated in 1929 with the introduction of a precise analytical method by Heidelberger and his associates in the United States, constituted the starting point of modern immunochemistry.

Increasing quantities of specific antigen[12] are added to a series of tubes containing a constant quantity, say 1 ml, of rabbit anti-ovalbumin serum. After incubation at 0 °C for 24 h or more to allow complete precipitation, the tubes are centrifuged; the precipitates are washed with 0.15 *M* NaCl at 0 °C and quantitatively transferred to micro-Kjeldahl tubes to measure the content of protein nitrogen. Alternatively, any other method of measuring protein content can be used, such as colorimetric methods (biuret, Folin-Ciocalteu, and others) as well as ultraviolet absorption at 280 nm.

It has been verified under these conditions that the quantity of precipitate increases progressively with the quantity of antigen added until a maximum is reached, from which point the quantity begins to decline by virtue of the formation of soluble complexes due to antigen excess. Representative data from an experiment of this type are shown in Table 7.4 and in Fig. 7.30.

Figure 7.30 shows that the specific precipitation curve comprises three distinct segments: an initial ascending portion, a

12 Heidelberger and Kendall first used pneumococcal polysaccharide as antigen, which has the advantage that it does not interfere in the determination of the antibody protein as N. This problem has since been solved through the use of radioactively labeled antigens

Ag	Precipitate (Ag+Ab)	Ab	Weight ratio Ab/Ag	Molar ratio Ab/Ag	Test of the Supernatants
9	156	147	16.2	4	Antibody excess
40	526	486	12.2	3	Antibody excess
50	632	582	11.6	2.9	Antibody excess
74	794	720	9.7	2.4	Neither Ag nor Ab
82	830	748	9.1	2.3	Trace of Ag
90 (87)[b]	826	739	8.5	2.1	Antigen excess
98 (80)	820	731	8.2	2	Antigen excess
124 (87)	730	643	7.4	1.8	Antigen excess
307	106	–	–	–	Antigen excess
490	42	–	–	–	Antigen excess

Table 7.4. Quantitative data of specific precipitation in the ovalbumin-rabbit anti-ovalbumin system[a]

[a] Addition of increasing amounts of ovalbumin to 1 ml of serum. Values expressed in µg N

[b] The values in parentheses correspond to the quantities of antigen in the precipitates, calculated by subtracting from the total Ag the quantity measured in the supernatant in the presence of a calibrated antiserum. The Ab/Ag molar ratio was obtained by dividing the weight ratio by the quotient of the molecular weights of the antibody and the antigen (160,000/40,000 = 4)

plateau corresponding to the precipitation maximum, and a descending terminal segment. These three segments are clearly delineated by examination of the supernatant from each reaction tube after centrifugation of the specific precipitates. Such tests can be performed either by means of the ring test in the proper capillary tubes, or by gel precipitation as indicated in the figure. Under these conditions, one can demonstrate that the initial (ascending) portion and the terminal (descending) portion correspond, respec-

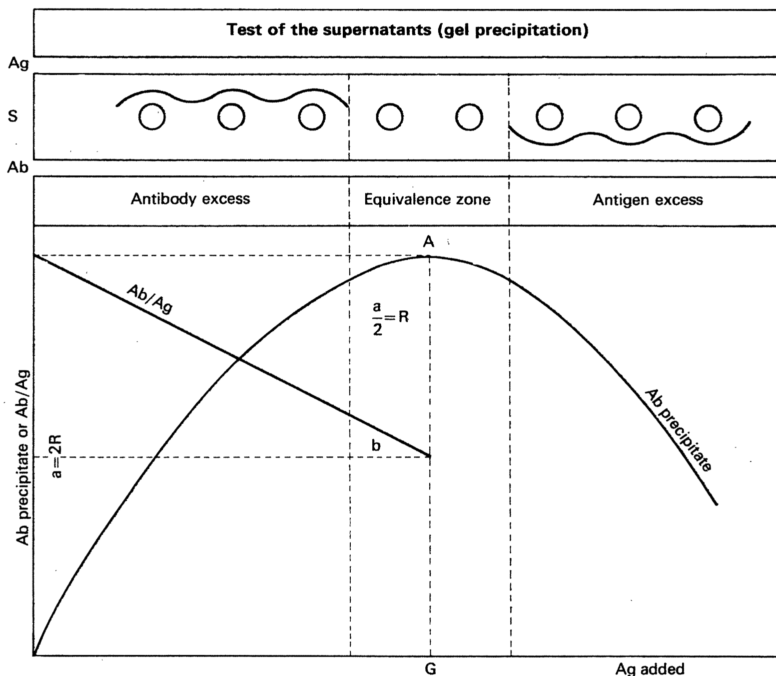


Fig. 7.30. Quantitative relationships in specific precipitation

tively, to antibody-excess and antigen-excess zones, whereas the plateau region has an excess of neither. The plateau region thus depicts an equivalence zone in which the antigen and antibody are in optimum proportions and are incorporated into the precipitate.
The curve just described exhibits only the suggestion of an inhibition zone in the area of antigen excess; since this fact is commonly observed with rabbit antisera, this type of curve is called "rabbit type" or "precipitin type." With anti-protein horse sera (e.g., against diphtheria toxin), the curve is different: it does not originate at 0 but rather from a positive abscissa value, and it has a characteristically bell-shaped form. Such antisera thus exhibit two inhibition zones, one for antibody excess and another for antigen excess. This type of curve is termed "horse type" or "flocculation type." In reality, horse antitoxin can exhibit both types of curves, depending upon the nature of the immunoglobulin with which it is associated – IgG (precipitin type) or IgGT (flocculation type).

Quantitative Relationships in Specific Precipitation. Figure 8.30 further shows that the antibody/antigen (Ab/Ag) weight ratio in the specific precipitates is a linear function of Ag, expressed by the equation

$$\mathrm{Ab}/\mathrm{Ag} = a - b(\mathrm{Ag})\,, \qquad (1)$$

in which a (intersection with the ordinate axis) and b (slope of the line) are constants proper to each antiserum.
From eq. (1) we derive

$$\mathrm{Ab} = a(\mathrm{Ag}) - b(\mathrm{Ag}^2)\,, \qquad (2)$$

which permits calculation of the Ab value for each dose along the precipitation curve. In the example in Table 8.4, $a = 15.8$ and $b = 0.083$, so that the equation of the serum is

$$\mathrm{Ab} = 15.8(\mathrm{Ag}) - 0.083 \times \mathrm{Ag}^2\,,$$

when Ag is expressed in μg N, or

$$\mathrm{Ab} = 15.8(\mathrm{Ag}) - 83 \times \mathrm{Ag}^2$$

when Ag is expressed in mg N.
The absolute quantity of antibody in a serum corresponds to the value of Ab for the dose that maximally precipitates Ag. This dose can be calculated easily by the formula $A_{max} = a/2b$, which can be deduced from the relationship $b = \mathrm{R}/\mathrm{Ag}_{max}$ by taking into consideration the finding that a is approximately equal to 2 R (whereby $\mathrm{R} = a/2$):

$$\mathrm{Ag}_{max} = \mathrm{R}/b = a/2\,b\,.$$

Ab_{max} can be calculated from the equation

$$\mathrm{Ab}_{max} = a(\mathrm{Ag}_{max}) - b \times \mathrm{Ag}_{max};$$

then, substituting $a/2\,b$ for Ag_{max},

$$\mathrm{Ab}_{max} = a(a/2\,\mathrm{b}) - b(a^2/4\,b^2) = a^2/4\,b\,. \qquad (3)$$

Applying eq. (3) to the antiserum in the example, we obtain

$$\mathrm{Ab}_{max} = \frac{(15.8)^2}{4 \times 0.083} = 752\,\mu\mathrm{gN}\,,$$

which is in close accord with the experimental value of 748 μg N (Table 8.4), corresponding to a slight antigen excess.
In eq. (1), the constant a (which equals 2 R) denotes the degree of reactivity of the antibody, since evidently the greater the value of R, the greater is the quantity of Ab that combines with an equivalent quantity of Ag. The value of b depends not only on the quality, but also on the quantity of Ab, because b is equal to $\mathrm{R}^2/\mathrm{Ab}_{max}$. The latter ratio is calculated from $b = \mathrm{R}/\mathrm{Ag}_{max}$, substituting $\mathrm{Ab}_{max}/\mathrm{R}$ for Ag_{max} (by definition, $\mathrm{R} = \mathrm{Ab}_{max}/\mathrm{Ag}_{max}$, where $\mathrm{Ag}_{max} = \mathrm{Ab}_{max}/\mathrm{R}$).
To compare the equations of various sera with respect to relative antibody qualities, it is necessary to eliminate the quantitative factor; this can be achieved by multiplying b by Ab_{max} to cancel out the denominator and make b equal to $\mathrm{R}^2/1$.

Antigen	Molecular weight	A=2R	Molar ratio	
			Equivalence	Extreme antigen excess
Ribonuclease	14,000	33	1,5	3
Ovalbumin	40,000	20	2,5	5
Serum albumin	60,000	15	3	6
Gamma globulin	160,000	7	3,7	7

Table 7.5. Molecular composition of the precipitate for different immune systems (rabbit antibody)

Let us consider, for example, two antisera whose equations are

I) $Ab = 21.4\ Ag - 101\ Ag^2$,

II) $Ab = 21.4\ Ag - 167\ Ag^2$.

The two sera are seemingly different, because for one of them $b = 101$ whereas for the other $b = 167$. However, if we reduce eqs. I and II to the level of 1 mg of antibody by multiplying each b value by the respective Ab_{max} (1.136 for I, and 0.685 for II), a single equation results, denoting the identity of the two antisera:

$$Ab = 21.4\ Ag - 114\ Ag^2 .$$

The quantitive study of the specific precipitation reaction furthermore permits calculation of the molecular composition of the precipitates in the different zones of the precipitation curve. Thus, for example, in the ovalbumin-antiovalbumin system, the Ab/Ag ratio in the zone of extreme antigen excess is approximately equal to 5, whereas in the zone of extreme Ab excess, that figure is closer to 20. Since the molecular weights of the reagents involved are 40,000 and 160,000 daltons, the molar ratio of the complex in the extreme antibody-excess zone is

$$\frac{20/160{,}000}{1/40{,}000} = 5,$$

indicating an $AgAb_5$ complex. In the equivalence zone, the formula of the complex is $AgAb_{2.5}$, and in the antigen-excess zone it is $AgAb$ or Ag_2Ab.

The molecular weights of the antigens, the average a (or 2 R) values, and the respective molar ratios in the equivalence and extreme antigen-excess zones for different systems are shown in Table 7.5.
The molar Ab/Ag ratio in extreme Ag excess is frequently adopted as an estimate of the minimum number of determinants on the surface of the antigen molecule – as a measure of the valence of the Ag. However, this is a minimum estimate, for obviously there can be determinants incapable of uniting with the antibody because of steric hindrance; besides this, since the Ab is bivalent, a single antibody molecule can unite with two determinants of the same antigenic molecule.

Applications of the Precipitation Reaction: Qualitative Precipitation

The precipitation in liquid medium, in the form of the ring test, was introduced at the beginning of this century for the identification of blood stains in forensic investigations. Such identification was also useful for the discovery of hemophagocytic vectors.
The qualitative precipitation reaction is also used to diagnose infectious diseases, e.g., in the postmortem diagnosis of anthrax (Ascoli's reaction), and to identify bacteria, e.g., the Lancefield groups and the M-type streptococci. Today, precipitin reactions are used in the gel-precipitation form for antigenic analyses.

Quantitative Precipitation

Quantitative Assay of Antibodies. The absolute quantity of precipitins can be determined, in terms of milligrams of protein or of N per milliliter of antiserum, by means of the Heidelberger–Kendall method, or by analysis of the precipitate obtained through the addition, to a proper constant volume of antiserum, of a quantity of antigen slightly greater than the equivalence dose (see Table 8.4).

Even without analysis of the specific precipitates, it is possible to make an approximation of the level of precipitins by means of the so-called supernatants method, with its basis in the prior determination of the value of R, i.e., of the Ag/Ab ratio in the equivalence zone. Thus, for example, if to 1 ml of antiserum it is necessary to add 50 µg N ovalbumin so that a slight antigen excess remains in the supernatant – knowing that the value of R for the system involved is 10 – we could conclude that the antiserum contains approximately 500 µg of antibody (in N) per milliliter.

In the Heidelberger–Kendall method, the serum is maintained constant and the antigen is added in increasing quantitites to determine the maximum quantity of precipitate (slight Ag excess). However, one can determine comparatively the relative antibody content in different sera through the use of labeled antigens, (e.g., with ^{125}I or ^{131}I), thus determining the percentage of Ag incorporated into the specific precipitate. In the so-called P-80 method, the point of reference for the comparison is the dilution of serum corresponding to 80% incorporation (20% antigen excess in supernatant); this reference point generally is situated at the point of maximum precipitation.

In another method introduced by Farr for the assay of nonprecipitating antibodies,[13] the soluble complexes are precipitated by the addition to each tube of an equal volume of saturated ammonium sulfate solution,

13 It was formerly thought that the precipitating antibodies were univalent, but equilibrium dialysis experiments have shown that such antibodies can be of low or high affinity. Possibly, the high-affinity bivalent antibodies, when incapable of precipitation, possess an anomalous distribution of electric charges or other alterations that make a linkage with antigenic determinants impossible because of steric hindrance

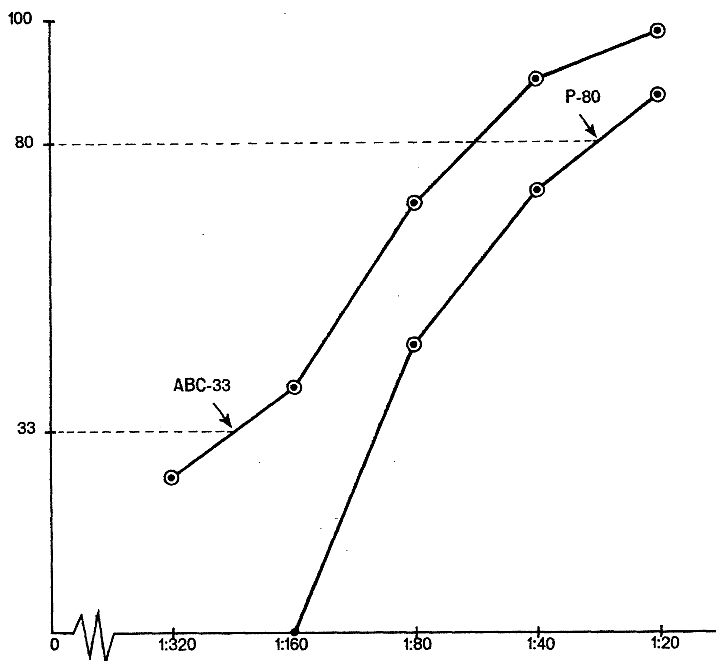


Fig. 7.31. Antibody concentrations measured by the ABC-33 and P-80 methods

and the precipitates are analyzed after careful washing to determine the percentage of fixed antigen (ABC method, antigen-binding capacity). The point of reference in this method was arbitrarily fixed at one-third of incorporation (ABC-33), which represents a considerable excess of antigens in the supernatant (two-thirds of the antigen added). In Fig. 7.31, the parallel results are depicted for the assay of an antiserum by the P-80 and ABC-33 methods.
Farr's method incontestably is of great interest in the study of nonprecipitating systems, but it requires rigorous standardization of experimental conditions and is limited by the fact that it can be applied only to systems in which the antigen is not precipitated by semisaturated ammonium sulfate, as in the anti-ovalbumin or anti-albumin systems.

Assay of Antigens. Quantitative precipitation can be utilized for assaying antigens, provided that a monospecific antiserum calibrated for the antigen in question, is available. An important application of this technique is the assay of gamma globulins in cerebrospinal fluid, of great importance in neurologic diseases – particularly in multiple sclerosis and neurosyphilis, formerly diagnosed by a nonspecific test (benzoin colloidal reaction).

Study of Cross-Reactions. Quantitative precipitation is also of special interest in the study of cross-reactions; it permits differentiation of those that are due to the existence of common antigenic determinants from those that result from the interaction of similar (but not identical) determinants and fairly well adapted antibodies.
The former case is exemplified by the reactions between chicken and duck ovalbumin, or between the S 3 and S 8 pneumococcic polysaccharides and their respective antisera. In this last example, which has been particularly well studied by Heidelberger and his associates, the cross-reaction is due to the existence in both of the polysaccharides of repeated units of cellobiuronic acid (1,4-glucuronoglucose or GnGl), in which S 3 is a linear polymer of GnGl, whereas S 8 is composed of alternate units of GnGl and of glucosyl-galactosyl (GlGa):

S 3 (GnGl)-(GnGl)-(GnGl)-(GnGl)-
S 8 (GnGl)-(GlGa)-(GnGl)-(GlGa)- .

Experimentation has confirmed what had been anticipated based upon the structure of the foregoing polysaccharides – that S 3, containing 100% GnGl residues per molecule, must precipitate better with anti-S 8 than does S 8 (only 50%) with anti-S 3.
The second type of cross-reaction embodies the reaction between similar antigenic determinants, e.g., m-azophenylsulfonate and m-azophenylarsonate, or 2,4-dinitrophenyllysyl and 2,4,6-trinitrobenzene.
In the case of common determinants, the heterologous reaction never reaches the maximum of the homologous reaction, with both curves exhibiting an equivalence zone clearly followed by an antigen-excess inhibition zone; however, if the cross-reaction occurs through similar determinants, the heterologous reaction curve can rise as high as that of the homologous reaction (when a sufficient quantity of antigen is added); however, it does not exhibit a distinct equivalence zone, and it forms soluble complexes only with difficulty because of the high degree of dissociation of the Ag-Ab complex.

Quantitative Inhibition of Specific Precipitation

Univalent antigens, incapable of precipitating, are nevertheless able to inhibit precipitation by the multivalent antigens. This inhibition can be studied quantitatively by mixing the antiserum first with an inhibitor and, second, with a multivalent antigen in a dose approximating equivalence. Thus, for example, if in the absence of inhibitor, the precipitation equaled 100 μg, whereas in the inhibitor's presence 80 μg was detected, we could say there was 20% inhibition. By comparing the inhibition percentages for differ-

ent amounts of inhibitor, a dose corresponding to 50% inhibition can be determined and adopted for use in determining the relative potency of different inhibitors.

Three important applications are derived from quantitative inhibition of the Ag-Ab reaction.

1. *Determination of the size of the antibody-combining site.* From the pioneering work of Kabat with the human anti-dextran antibodies and dextran, it can be concluded that the "cavity," or combining site, of the antibody corresponds to a chain of six or seven glucose molecules. This conclusion resulted from inhibition experiments with different oligosaccharides of isomaltose (IM 2 to IM 7), in which it was verified that maximum inhibition was obtained with isomaltohexose (IM 6) or with isomaltoheptose (IM 7). For different antisera, the relative inhibitive powers of the different oligosaccharides were variable, and this gave rise to an important additional conclusion, namely, that there was a heterogeneous population of antibodies with "cavities" of diverse sizes up to a maximum corresponding to IM 6 or IM 7. Experiments performed with other systems confirmed the data initially obtained with the dextran-antidextran reaction, that is, the antibody-combining site was capable of accommodating, at a maximum, six to seven glucose molecules or five to seven amino acids, with dimensions corresponding approximately to $34 \times 17 \times 7$ Å. The study of the dextran-antidextran system permitted further interesting inferences concerning the combining energies of the different groups of antigenic determinants. Based upon the proportionality existing between inhibitory potency and combining energy (ΔF^0), it was possible to evaluate the percentage contribution of each glucose to the total combining energy of IM 6. Although the first glucose (from the nonreductive end) contributed 40%, and the first three 90%, the increase from that point on was minimal (2%–5%). The group that contributed the greatest percentage of the total combining energy of the antigenic determinant was termed the "immunodominant group."

2. *Determination of the structure of the antigenic determinant.* Quantitative inhibition is also an important method for determining the structure of the antigenic determinants. For example, the A, B, and H determinants of the substances of the AB0 blood-group system are inhibited, respectively, by N-acetyl-D-galactosamine (A), by D-galactose (B), and by D-fucose (H), indicating that these sugars are the immunodominant groups of the determinants in question. The structure of the A determinant was investigated further through quantitative inhibition with the following oligosaccharides obtained through acid or alkaline hydrolysis of the A substance:

α-D-GalNAc-(1-→3)-β-D-Gal-(1-→3)-D-GNAc

```
                        α-L-Fuc-(1-2)
                              |
α-D-GalNAc-(1-→3)-β-D-Gal-(1-→4)-β-D-GNAc-R;
```

in which GalNac = N-acetyl-D-galactosamine; Gal = galactose, GNAc = N-acetyl-D-glucosamine, Fuc = fucose, and R is a radical resulting from the reduction of galactose. The reduced pentasaccharide was shown to be a more potent inhibitor than the trisaccharide, and the results of the quantitative inhibition taken together have permitted elucidation of the structure of the A determinants.

In addition to inhibition of specific precipitation, cross-reactions also can be used for the study of the structure of antigenic determinants. Using cross-reactions with antisera capable of reacting with antigens of known structure, Heidelberger studied the reactions of numerous polysaccharides with different type-specific horse sera. He was thus able to draw a conclusion concerning the unknown structures of those substances. For example, cross-reactions with anti-S 2 could be due to residues of glucose, ramnose, or glucuronic acid; however, in the particular case of the reaction with acacia (gum arabic), it was

possible to demonstrate that the reaction occurred with glucuronic acid, which otherwise constituted the immunodominant group of the S2-anti-S2 interaction. The anti-S14 serum reacts with polyglucoses as well as with terminal residues of galactose, which condition the cross-reaction with the substances of the AB0 group system, as well as with certain gums and mucilages of vegetable origin.

3. *Radioimmunoassay*. This type of assay is extremely sensitive. It permits measurement of antigen quantities at the level of picograms, based upon the competitive action between an unlabeled antigen (Ag) and the same antigen (Ag^x) labeled with a radioactive isotope (usually ^{125}I) in the formation of immune complexes in the presence of a limited quantity of specific antibody (Ab), which is linked covalently to an insoluble matrix (e.g., particles of Sephadex), or in the form of an insoluble anti-Ab complex (coprecipitation double-antibody system).

In a hypothetical example, if to five molecules of bivalent antibody (10 combining sites) variable quantities of unlabeled antigen are first added, followed by a fixed dose of labeled antigen (10 molecules), the percentage of bound radioactivity (or the bound Ag^x/free Ag^x ratio) would decrease, as illustrated in Fig. 7.32 and in the graph of Fig. 7.33. Upon comparision with a reference curve obtained from an Ag solution of known concentration, one can determine, by the decrease in radioactivity, the quantity of antigen present in a solution of unknown concentration.

Radioimmunoassay has been used with excellent results in the detecting and assaying of peptides and hormones (steroids, insulin, growth hormone, ACTH, gonadotropin and chorionic gonadotropin, follicle-stimulating and luteinizing hormones, lactogenic, placental, and other hormones); of certain tumoral antigens (α-fetoprotein, carcinoembryonic antigen); of drugs (digoxin, morphine); of viral antigens (Hb); and of immunoglobulins (IgE).

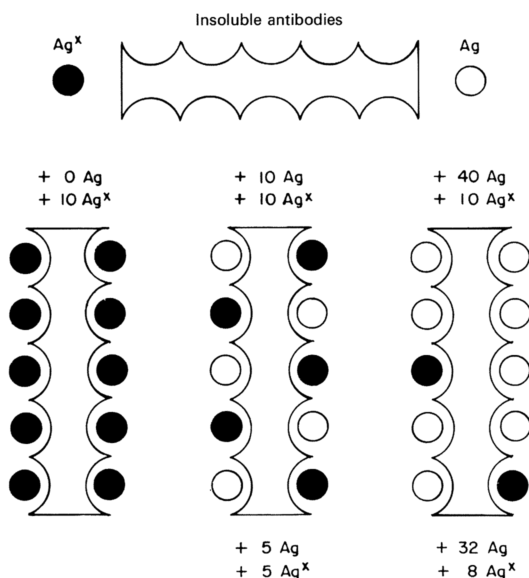


Fig. 7.32. Diagrammatic representation of the principle of the radioimmunoassay

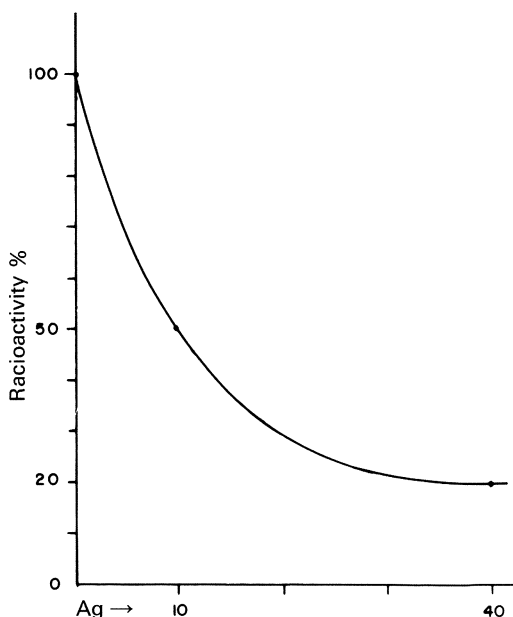


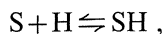
Fig. 7.33. Curve based upon the hypothetical example shown in Fig. 7.32: The drop in bound radioactivity due to the competitive action of Ag and Ag^x is approximately sigmoidal. When the antigen quantities are represented by logarithms along the abscissa, a straight line is obtained whose slope facilitates the calculation of the concentration of Ag in an unknown solution (once the standard curve is established)

Enzyme-linked Immunosorbent Assay (ELISA)

This method is in principle entirely analogous to direct or indirect immunofluorescence techniques. Instead of a fluorescent dye, an enzyme is conjugated to an antibody; horseradish peroxidase is most commonly used as enzyme, but virtually any enzyme can be employed as long as it is soluble, stable, and not present in biological fluids in quantities that would interfere with serum determinations. The test can be used to measure either antigen or antibody and is analogous to the radioallergosorbent test (RAST) (see p. 275). To measure antibody, antigen is fixed to a solid phase, incubated with test serum, and then reacted with enzyme-tagged anti-immunoglobulin. Enzyme activity adherent to the solid phase is measured spectrophotometrically, and then related to amount of antibody bound.
To measure antigen, antibody is bound to the solid phase, a test solution containing antigen is added, and then a second enzyme-labeled antibody is added. This test requires that at least two determinants are present on the antigen. Advantages of the enzyme immunoassay include sensitivity (ng/ml range), simplicity, stability of reagents, lack of radiation procedures, and that it is relatively inexpensive.

Quantitative Study of the Hapten–Antibody Reaction

The hapten–antibody reaction is a reversible reaction:



$$S + H \rightleftharpoons SH ,$$

whose association constant can be calculated by the law of mass action:

$$K = \frac{(SH)}{(S)\,(H)} ,$$

in which S represents the combining site of the antibody and H represents the hapten. If n represents, the valence of the antibody,

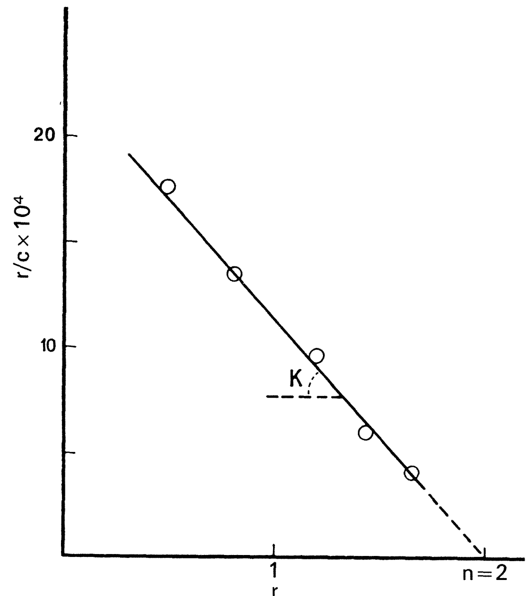


Fig. 7.34. Scatchard's equation (r/c vs. r)

r, the number of molecules of H bound per molecule of antibody, and c, the concentration of H, then

$$K = \frac{r}{(n-r)\,c} ,$$

for $r = SH$ and $(n-r)$ is equivalent to S. From the preceding equation,

$$r/c = K_n - K_r \text{ (Scatchard's equation)} ,$$

for the equation of the line that expresses the variation of r/c as a function of r in which K_n is the intersection with the ordinate and K is the slope (Fig. 7.34).
For the zero value of the ordinate, the intersection of the line with the abscissa corresponds to a value for r equal to n (for $r/c = 0$, $K_n = K_r$).
It thus becomes possible to calculate K and n if we know the values of r and c, which are obtained experimentally by special techniques (equilibrium dialysis, fluorescence quenching), described below.
Frequently, however, the variation r/c versus r is not linear in its full extension, so that

the values for K are not uniform.[14] It is convenient, therefore, to measure the affinity of antibodies by expressing them as a function of a median value K_o (intrinsic association constant), which corresponds to the occupation of half of the sites S ($r=1$). This value can be calculated as K_o equals $1/c$, for if in Scatchard's equation we make n equal to 2 (bivalent antibody) and r equal to 1, we obtain

$$1/c = 2K - K = K^\circ .$$

The value of K^o ($1/c$) can also be estimated graphically as the middle of the K_n intersect, or the r/c value corresponding to $r=1$.

Equilibrium Dialysis. One milliliter of purified antiserum (S) is placed in a small cellophane tube. The tube is closed with a knot and is placed inside a glass flask containing 1 ml 0.15 *M* NaCl to which is added a known concentration of hapten (H). The mixture is agitated gently until the concentration of H in the exterior liquid reaches a constant value (equilibrium). The initial and final distributions of the molecules of hapten and antibody are illustrated in Fig. 7.35.

Where H is a colored compound, its concentration can be measured spectrophotometrically. Otherwise, one should use a solution of labeled hapten. The experiment should include controls for the nonspecific adsorption of the hapten on the normal gamma globulins.

Knowing the value for the free concentration of H (c), one can determine by subtraction how many moles of H have combined with the antibody and, consequently, r can then be calculated, where r equals the number of moles of H fixed per mole of antibody. Table 7.6 illustrates the simplified protocol of an experiment of this type.

If one supposes that the number of moles of H in the exterior phase of the tube in equilibrium is equal to 1.11×10^{-5}, then the quantity of free H in both sides is equal to 2.22×10^{-5} *M*. If tube 1 indicates nonspecific adsorption of 10% to the membrane,

14 To correct the effect of the heterogeneity of the antibodies in relation to the value of K, Sips' function (similar to Gauss's function) is used, which leads to the equation
$r/(n-r) = (K \times c)^a$,
in which a represents the index of heterogeneity. For $a=1$ (absence of heterogeneity), the preceding equation is transformed into Scatchard's equation

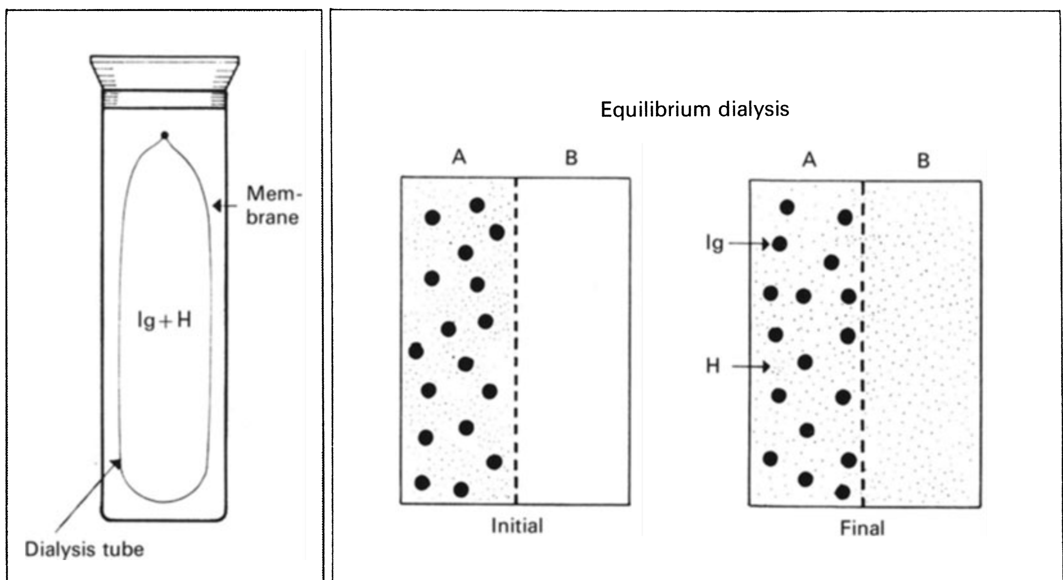


Fig. 7.35. Equilibrium dialysis

Table 7.6. Simplified protocol of an equilibrium dialysis experiment

Tube no.	Interior	Exterior
1	0.15 M NaCl	5×10^{-5} M H
2	4×10^{-5} S	5×10^{-5} M H

this value must be corrected by $(2.22+0.22)\times10^{-5}$ or to 2.44×10^{-5}, and the quantity of H fixed by S can be estimated at (5−2.44), i.e., at 2.56×10^{-5} M.
Since the total amount of antibody added is known (4×10^{-5} M), $r=2.56/4=0.640$. K° ($1/c$) is equal to the reciprocal of 2.44×10^{-5}, or 0.41×10^{5} M.

Fluorescence Quenching. The hapten–antibody reaction tends to extinguish part of the fluorescence that normally is exhibited by the immunoglobulin when irradiated with ultraviolet light (280 nm). This fluorescence quenching is due to residues of tryptophan found in the combining site of the antibody, which, when covered by the hapten, transmit to the latter the energy it has absorbed and that it should have emitted in the form of fluorescent light (330–350 nm).
The technique of fluorescence quenching is advantageous in that it can be performed rapidly and requires only tiny quantities of antiserum; however, to be applied to unknown systems, it must always be run in parallel with an equilibrium dialysis, which is the standard method.

Thermodynamics of the Hapten–Antibody Reaction. Energy is the capacity to produce work, whereas so-called free energy (F) is that which produces maximum work. The value of F cannot be measured in absolute terms; however, it is possible to measure the positive or negative variations of F that occur when there are transformations in a system. In exothermic reactions – those that liberate energy – F has a positive value; in endothermic reactions – the inverse – energy must be furnished and F has a negative value.[15]
Free energy F is an exponential function of the association constant:

$$K^\circ = e^{-\Delta F/RT},$$

where

$$\Delta F^\circ = -RT\times\ln K^\circ = -4.57\times T\times\log K^\circ.$$

For example, if the determination of K°, at the temperature of 25 °C (298 T) results in the value 1.57×10^5, the variation of free energy can be calculated as

$$\Delta F^\circ = -4.57\times298\times\log(1.57\times10^5) = -7.09\ \text{kcal/mol}.$$

For different Ag–Ab systems, different values for $-\Delta F^\circ$ are encountered from 6 to 11 kcal/mol, corresponding to values for K° between 1×10^4 and 1×10^9.

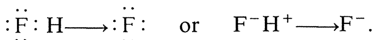
Intermolecular Forces in the Antigen–Antibody Reaction

The union of the antibody with the antigen, as with the union of an enzyme with the substrate, depends essentially upon the complementary adaptation of their tridimensional structures. This stereometric adaptation results in the mutual attraction of the opposed surfaces through short-reaching covalent forces that are inoperative between molecules not in sufficient proximity.
An analogous example is the glueing together of two fragments of a broken piece of china; after a layer of glue is brushed on the surface of each fragment, the two pieces

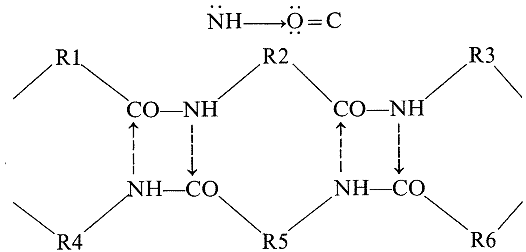
15 Free energy (F) represents merely one part of the total energy (enthalpy or H); the other part includes a degraded form of energy associated with the disorder of the system, called entropy (S): $\Delta H=\Delta F+T\Delta S$. To calculate the value of enthalpy (and, by subtraction, that of entropy), it is necessary to determine the association constants for two temperatures and to utilize Van t'Hoff's formula: $\Delta H = R\times T_1\times T_2\times\ln(K_2/K_1)/(T_2-T_1)$

must be held closely fitted together for a period of time. In the antigen–antibody reaction, the glue is represented by attractive forces which operate at the level of the combining sites of the reagents, subject to the complementary adaptation of their surfaces. The following are the intermolecular forces that come together in the Ag–Ab union:

1. *Ionic or coulomb forces* result from the electrostatic attraction between ions of opposite charges, e.g., COO^- and NH_4^+.
2. *Polar attraction forces* occur between dipoles and between ions and dipoles. A particular case is represented by the hydrogen bond, in which H, linked covalently to an electronegative atom, is attracted by a pair of unshared electrons of another electronegative atom:



Though they are weak (3–7 kcal/mole), the numerous hydrogen bridges between the NH and CO groups of peptide bonds play an important role in maintaining the secondary structure (alpha helix) of the proteins:



3. *Van der Waals forces* are the weakest forces (1–2 kcal/mol), operating only in a short radius of action, where the proximity of the molecules results in the induction of fluctuating charges originating from the attrac-

Table 7.7. Relative sensitivities of various immunologic techniques

Method	Vol. of serum used in test (ml)	Sensitivity Limit	
		μg N-Ab/ml	μg N-Ab
Specific precipitation			
Qualitative:			
Ring test	0.1	2–5	0.2–0.5
Gel diffusion			
Oudin	0.2	2–5	0.4–1.0
Ouchterlony	0.1	5–10	0.5–1.0
Preer	0.01	5–10	0.05–0.1
Quantitative:			
Micro-Kjeldhal			20 (20–100)
Mod. Markham			10 (10–100)
Biuret (550 nm)			20 (20–100)
Folin-Ciocalteu (750 nm)			2 (2–30)
UV absorption (277 or 287 nm)			5 (5–100)
Passive hemagglutination	0.1	0.03–0.06	0.003–0.006
Complement fixation	0.1	0.5–1.0	0.05–0.01
Diptheria toxin neutralization (Römer-Frazer test)	0.1	0.01–0.04	0.001–0.004
Passive cutaneous anaphylaxis (rabbit-antibody, guinea pig skin)	0.1		0.003–0.006
Radioimmunoassay; ELISA		μg-ng range	ng-pg range

tion exercised by the nucleus of one of the atoms upon the electrons of the external orbit of the other atom and vice versa. Also called London forces, these intermolecular forces do not appear to play an important role in the Ag–Ab union.

4. *Apolar or hydrophobic bonds* occur in aqueous solution between apolar groupings. They work by virtue of their property of excluding the ordered network of H_2O molecules that are interposed between the dissolved molecules, furthering the approximation of these to the action radius of short-reaching forces – Van der Waals forces in particular. The hydrophobic residues of certain amino acids (alanine, phenylalanine, leucine, isoleucine, tyrosine, tryptophan, methionine) play a relevant role in the tertiary structure of the proteins.

Comparative Sensitivities of Serologic Techniques

The various methods for detecting antibodies exhibit greatly differing sensitivities, as indicated in Table 7.7, in terms of the concentration or of the absolute quantity of antibody that the respective reactions are capable of disclosing.

The determination of the minimum quantity of antibody evidenced by a reaction depends upon the underlying practical conditions, or more specifically, upon the volume of serum utilized and the inherent peculiarities of the individual test. Thus, for example, passive hemagglutination is capable of detecting 0.003–0.006 μg N of antibody in tests that utilize 0.1 ml of serum.

In cutaneous anaphylaxis (PCA) however, in which 0.03 μg of Ab-N can be detected, a serum that contains 0.3 μg Ab-N does not exhibit the PCA reaction because of the blocking action of normal immunoglobulins.

With the radioimmunoassay, serum at a dilution up to 10^{-6} can be used, and antigens such as corticosterone can be measured with precision in amounts as small as 5 pg (1.5×10^{-14} M).

References

Ackroyd FF, Turk JL (eds) (1970) Immunological methods. Blackwell, Oxford

Arquembourg PC, et al. (1970) Primer of immunoelectrophoresis. Ann Arbor Humphrey, Ann Arbor/Mich.

Barret JT (1970) Textbook of immunology. An introduction to immunochemistry and immunobiology. CV Mosby, St. Louis

Berson SA, Yalov R (1968) General principles of radioimmunoassay. Clin Chim Acta 22:51

Burrows W (1951) Endotoxin. Ann Rev Microbiol 5:181

Campbell DH et al. (1970) Methods in immunology (2nd ed). WA Benjamin, New York

Chase MW, Williams CA, jr (1971) Methods in immunology and immunochemistry, vols I and II. Academic Press, New York

Coombs RRA, et al. (1961) The serology of conglutination and its relation to disease. Blackwell, Oxford

Coons AH (1958) Fluorescent antibody methods. In: Danielli JF (ed) General cytochemical methods. Academic Press, New York

Crowle AJ (1961) Immunodiffusion. Academic Press, New York

Davis BD, et al. (1967) Microbiology (2nd ed) Chaps 12–18. Harper & Row (Hoeber), New York

Dubos RJ, Hirsch JG (eds) (1965) Bacterial and myotic infections of man, 4th ed. Lippincott, Philadelphia

Eisen HN (1964) Equilibrium dialysis for measurement of antibody-hapten affinities. Methods in Med Res Yearbook 10:16

Fahey JL, McKelvey EM (1965) Quantitative determination of serum immunoglobin in antibody agar plate. J Immunol 94:84

Furth R van, et al. (1972) The mononuclear phagocytic system, monocytes and their precursor cells. Bull WHO 46:845

Givol D (1970) Procedures for sensitive radioimmunoassay. Int Atom Energy Agency 1245:15/72

Grabar P, Burtin P (1960) L'analyse immunoeléctrophorétique. Masson, Paris

Heidelberger M (1947) Immunochemistry of antigens and antibodies. In: Cooke (ed) Allergy in theory and practice. Saunders, Philadelphia

Heidelberger M (1969) Quantitative absolute methods in the study of antigen-antibody reactions. Bact Rev 3:49

Holborrow EJ (ed) (1970) Standardization in immunofluorescence (2nd ed). Blackwell, Oxford

Kabat EA (1961) Kabat and Mayer's Exp Immunochemistry, 2nd ed, Chaps 1–12. CC Thomas, Springield/Ill.

Kabat EA (1971) Einführung in die Immunchemie und Immunbiologie. Springer, Berlin Heidelberg New York

Kabat EA (1976) Structural concepts in immunology and immunochemistry, 2nd ed. Holt, Rinehart & Winston, New York

Karush F (1962) Immunological specificity and molecular structure. Adv Immunol 2:1
Kwapinski JBG (1972) Methodology of immunochemical and immunological research. Wiley, Chochester
Nairn RC (1969) Fluorescent protein tracing. Livingstone, Edinburgh
Oakley CL (1954) Bacterial toxins. Ann Rev Microbiol 8:411
Osler AG (1968) Quantitative study of complement fixation. Bact Rev 20:166
Ouchterlony O (1958/1962) Diffusion-in-gel methods for immunological analysis. Progr Allergy 5:1/6:30
Oudin J (1952) Specific precipitation in gels and its application to immunochemical analysis. In: Methods in Med Res Yearbook 5:335
Pappenheimer A, Jr (1948) Proteins of pathogenic bacteria. Adv Protein Chem 1:69
Pinckard RN, Weir DM (1973) In: Weir DM (ed) Handbook of exp immunology, 2nd ed. Blackwell, Oxford
Prevot AR (1950) Hemolysines et antihemolysines bactériennes. Le Sang 21:565
Raynaud M (1959) Heterogeneity of diphtheria antitoxin. In: Mechanisms of hypersensitivity. Little, Brown and Co, Boston
Relyveld EH (1959) Toxine et antitoxine diphthériques. Etude immunologique. Harmann, Paris
Schmidt H (1931) Die Praxis der Auswertung von Toxinen und Antitoxinen. Gustav Fischer, Jena
Staub AM, Raynaud M (1971) Cours d'Immunologie générale et de Sérologie de l'Institut Pasteur. CDU, Paris
Thomas L (1954) The physiological disturbances produced by endotoxins. An Rev Physiol 16:467
Van Heyningen WE (1954) Toxic Proteins. In: Neurath, Bailey (eds) The Proteins, vol II. Academic Press, New York
Westphal O (1957) Pyrogens. In: Second Macy Conf on Polysaccharides in Biology. Macy Foundation, New York
Zwilling R (1977) Immunologisches Prakticum. Gustav Fischer, Stuttgart