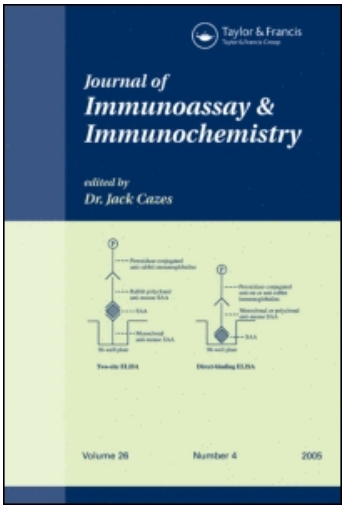

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Precipitation and Agglutination

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I. PRECIPITATION

A. Introduction

Immune precipitation is the formation of insoluble complexes as a result of the specific interactions between antigen molecules and the corresponding antibody molecules, both in aqueous solution. Usually, the largest amounts of precipitate are obtained when soluble antigens and antibodies are present in approximately equal concentrations. Therefore, to determine the concentration, or just the presence of antibody, precipitation requires considerable amounts of antigen; the method usually does not allow the detection of less than microgram quantities of antibody; that is, it is about 1000 times less sensitive than agglutination. Contrary to agglutination, divalent IgG (and not decavalent IgM) is the immunoglobulin with the strongest precipitating power.

A major breakthrough that caused the diversification of immune precipitation into a variety of different and powerful analytical methods was the development of precipitation in gels. This approach gave rise to methods permitting, for example, the distinction of small differences between antigenic sites, and the characterization of 100 or more different blood serum proteins.

B. Solubility of Antigens and Antibodies

The aqueous solubility of proteins and polysaccharides depends on their hydrophilicity. The electrical surface potential (ζ-potential) of proteins and polysaccharides contributes only to their solubility, to any significant degree, in the most highly charged proteins, such as serum albumin: $\zeta - 18$ mV). For proteins and polysaccharides with ζ-potentials of the order of 14 mV or smaller, the electrostatic contribution to solubility at physiological pH and ionic strength becomes negligible. The ζ-potential of immunoglobulins is of the order of only a few millivolts. Thus, in all cases of antigen–antibody (Ag–Ab) complex solubility, electrostatic forces play virtually no role, with the rare, but possible exception, of Ag–Ab complexes with a strong Ag excess and with Ags with an unusually high ζ-potential.

The solubility (s) of molecules of proteins, polysaccharides, Ag–Ab complexes, and other biopolymers depends on the interfacial (IF) free energy of interaction between such biopolymer molecules (i) when immersed in water (w):

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$$\Delta G^{IF}_{iwi}/kT = \ln s^* \quad (1)$$

where $kT = 4.045 \times 10^{-21}$J, at $T = 293°$ K, and

$$\Delta G^{IF}_{iwi} = -2\gamma_{iw} \quad (2)$$

where γ_{iw} is the interfacial tension between the biopolymer and water; its value can be obtained from contact angle measurements on layers of concentrated protein (van Oss et al., 1988; van Oss, 1993). In Eqs. (1) and (2), ΔG^{IF}_{iwi} is expressed in energy units per surface area: for Eq. (2), ΔG^{IF}_{iwi} as well as γ_{iw} are normally expressed in units of milliJoules per square meter (mJ/m^2); however, in Eq. (1), ΔG^{IF}_{iwi} should be expressed in energy units per molecule pair; to that effect one needs to know the "contactable surface area," S_c, for such molecule pairs. Thus, when ΔG^{IF}_{iwi} in Eq. (1) is expressed in units of energy per unit surface (e.g., mJ/m^2), Eq. (1) becomes:

$$\Delta G^{IF}_{iwi} \cdot S_c/kT = \ln s \quad (1A)$$

For significant aqueous solubility ΔG^{IF}_{iwi} must have a positive value, because of (1) the fairly large values of S_c for polymers and biopolymers, and (2) the strong effect of the hydrophobic attraction, which usually manifests itself in aqueous media when $\Delta G^{IF}_{iwi} < 0$ (van Oss and Good, 1992; van Oss, 1993). When $\Delta G^{IF} > 0$, solubility should prevail regardless of the size of the contactable surface, S_c (i.e., regardless of the size of the protein or other biopolymer). Nevertheless, when large Ag–Ab complexes are formed between soluble Ag and soluble Ab molecules, insolubility prevails, apparently as a consequence of the increase in molecular size.

C. Origin of the Insolubility of Large Antigen–Antibody Complexes

As a consequence of the outward orientation of the more hydrophilic sites of proteins, when immersed in water in their native configuration, their *average* ΔG^{IF}_{iwi} value tends to be greater than zero. However, there usually also is a number of somewhat more hydrophobic patches on most proteins. When such slightly hydrophobic patches approach each other, $-1\ kT < \Delta G^{IF}_{iwi} < 0$. In such cases, solubility still prevails for single protein molecules. However, when complex formation causes an increase in their molecular size, the situation can change *locally* to $\Delta G^{IF}_{iwi} < -1\ kT$, owing to an increase in the total contactable surface area, S_c, of the now more numerous hydrophobic patches. For instance, if the free energy of attraction between opposing slightly hydrophobic patches on two protein molecules is $\Delta G^{IF}_{iwi} = -2.0\ mJ/m^2$, and if each of these patches has a contactable surface area, $S_c = 1\ nm^2$, then, in units of kT, $\Delta G^{IF}_{iwi} = -0.5\ kT$, which still denotes solubility. But when complexes arise which can interact in such a manner that, for example, 12 such patches can interact, then a total contactable surface area, $S_c = 12\ nm^2$ is treated, resulting in $\Delta G^{IF}_{iwi} = -6\ kT$, indicating incipient *insolubility*. Thus, soluble proteins with a given surface energy can form larger complexes which still have the same surface energy per unit surface area, but which now have a *larger surface area*. From a given size on, these larger complexes can become insoluble even without the need for a change in surface properties. The critical radius for incipiently insoluble immune complexes is of the order of 100 nm (Easterbrook-Smith, 1993).

In addition, however, upon formation of Ag–Ab complexes, generally some change in surface properties occurs at the same time, for two reasons: (1) Fab is more hydrophilic than Fc (van Oss et al., 1975, pp. 38, 39), so that on bond formation between paratope and epitope, hydrophilic sites are lost on the Ab; and (2) epitopes tend to be among the more hydrophilic sites on the surfaces of proteins and peptides, so that when epitopes on an Ag become masked

*For a List of Abbreviations, Symbols and Units, see Chapter 23.

through binding to the corresponding paratopes, the complex thus formed also becomes more hydrophobic on the part of the Ag. A general tendency therefore exists for the surfaces of Ag–Ab complexes to be less hydrophilic than their original single components (free Ag and Ab). In addition, as discussed above, the total contactable surface area, S_c, of the somewhat more hydrophobic (and thus potentially attractive) sites, increases. Thus, the larger the complex, the lower its solubility. Very small complexes also are more soluble because these occur in either Ag or Ab excess: In Ag excess, more hydrophilic epitopes are still exposed and, in Ab excess, more hydrophilic Fab moieties remain exposed. Thus complexes formed from perfectly soluble Ag and Ab molecules become insoluble when they grow beyond a certain size, without any need for a change in structure or conformation of either Ag or Ab. In other words, for most biopolymer or biopolymer complexes, an increase in size suffices to cause insolubilization. The critical size in most cases is between molecular weights of 10^6 and 10^7. Large complexes of that type are increasingly prone to further spontaneous association, yielding particles of about 5 μm in diameter, which subsequently combine in larger clumps, both through specific rearrangements between antibody-active sites and antigenic determinants and *via* aspecific protein–protein interactions, mainly through hydrophobic interactions. It can take several hours before immune precipitate particles attain their ultimate size. The fact that immune complexes consist for an important part of immunoglobulins (especially IgG), which are among the less soluble blood serum proteins, also contributes to their low solubility.

It is well-known that IgM class Abs do not form immune precipitates as easily as IgG class Abs, notwithstanding their greater size and higher valency. This must be ascribed to the facts that (1) IgM does not expose its centrally located more hydrophobic Fc chains after binding to Ags, and (2) IgM is more hydrophilic than IgG (van Oss et al., 1985a).

The solubility of biopolymers in water can be increased by adding exposed OH and especially O groups. Such hypersoluble biopolymers, when used as antigens, tend to become toleragenic, as exemplified by the insertion of poly(ethylene glycol) groups into protein Ags (Sehon, 1989; Sehon et al., 1987).

D. Enhancement of Insolubilization of Antigen–Antibody Complexes

There are a number of approaches that can be used to enhance the insolubilization of biopolymers and, in particular, of soluble Ag–Ab complexes.

1. Flocculation Through Dehydration

The procedure of flocculation through dehydration is often used in "salting out" (van Oss et al., 1985a), usually through the admixture of 1 to 4 *M* $(NH_4)_2SO_4$. When applied to soluble immune complexes (Farr, 1958), this method is only effective in cases where there is no risk of dissociation of Ag–Ab complexes at high salt concentrations.

2. Flocculation Through Phase Separation

The procedure of flocculation through phase separation consists of the admixture of 2–10% polyethylene glycol (PEG) (Schultze and Heremans, 1966). Usually PEG (Mw ≈ 6000) concentrations of 2–5% are best suited for precipitating soluble immune complexes, but concentrations up to 7.5% PEG may be preferable for very small complexes (Albini et al., 1984). For the detection of medium- and low-avidity anti-DNA Abs (e.g., in systemic lupus erythematosus), PEG precipitation is the method of choice, because complexes formed with this type of Abs are prone to dissociate in concentrated salt solutions (Smeenk and Aarden, 1980; de Groot et al., 1980; Smeenk et al., 1982; 1983; van Oss et al., 1985b). One drawback of both flocculation approaches is that aspecifically aggregated proteins may coflocculate under the influence of

$(NH_4)_2SO_4$ or PEG and thus could contaminate the flocculated Ag–Ab complexes (Albini et al., 1984).

E. Precipitation in Tubes

1. Antigen/Antibody Ratios and Nonstoichiometry

Immune precipitation in aqueous media in test tubes is one of the oldest in vitro methods for obtaining quantitative data on the amount of specific antibody present in an antiserum. From precipitation in tubes, it became clear that only at an "optimal" Ag/Ab ratio a maximum amount of precipitate ensues; at slight Ab *or* Ag excess, no visible precipitate develops at all, although in these cases the formation of soluble complexes can be detected (e.g., by analytical ultracentrifugation). At the "optimal" Ag/Ab ratio no dissolved Ag *or* Ab can be detected in the supernatant liquid by ordinary means. Because the Ag–Ab reaction is reversible, one can detect the presence of very small amounts of free Ag and Ab in the supernatant even at optimal Ag/Ab ratios (e.g., by means of radioiodinated reagents; see van Oss and Walker, 1987). One important conclusion emerges from these observations: *The Ag–Ab reaction is not stoichiometric, but can take place at a wide range of Ag/Ab ratios*. From Ag–Ab precipitates obtained at "optimal" (i.e., equivalence) ratios, one can obtain the valency ratios of Ag and Ab only by a fairly rough approximation; it is only at Ag excess that one can determine the exact valency of the Ab (Edberg et al., 1972), while conversely, the exact valency of the Ag is obtainable only at Ab excess.

As immune precipitation requires the formation of large Ag–Ab "networks" (van Oss, 1979), it is essential if one wishes to achieve precipitate formation, that Abs as well as Ags be plurivalent, or at least divalent (as are most Abs). Precipitation is therefore impossible with single monoclonal Abs (MAbs) which, even though divalent, are directed against only one specificity (and thus, usually, against only one valency) of the Ag (Molinaro et al., 1984).

2. Automated Precipitation Methods

Contrary to automated agglutination and hemagglutination methods, in which the nonagglutinated particles or cells are quantitated (see Sec. II), in automated immune precipitation methods only the amount of precipitated material is measured. That amount of precipitated material is measured by light scattering (either at angles of 90°, or at fairly sharp forward-scattering angles: nephelometry). Great care should be taken in automated precipitation to avoid working under conditions of an excess of either Ab or Ag, to obviate decreased precipitate formation under those conditions (see above), by meticulous preliminary titrations of the Ab reagents as well as of the various Ag controls.

F. Double-Diffusion Precipitation in Gels

1. Cause of Line Formation

Almost half a century ago, Ouchterlony (1949; 1968) developed the technique in which solutions of Ag and Ab are deposited in separate wells punched in an agar gel slab and allowed to diffuse toward each other. At their place of encounter, a sharp, usually somewhat curved Ag–Ab precipitate line is formed. That precipitate line actually is a precipitate membrane, or barrier, seen from above. The salient property of that precipitate membrane or barrier is its specific impermeability for the dissolved Ag and Ab molecules situated on either side of it (van Oss and Heck, 1961). The Ag and Ab molecules that are unrelated to the Ag and Ab that formed the precipitate can freely pass that precipitate; this is why precipitate lines formed with identical Ag–Ab systems fuse, whereas such lines made by two unrelated Ag–Ab systems cross (van Oss and Heck, 1961; van Oss, 1968; 1984a) (Fig. 1). The specific impermeability of the precipitate barrier formed by a

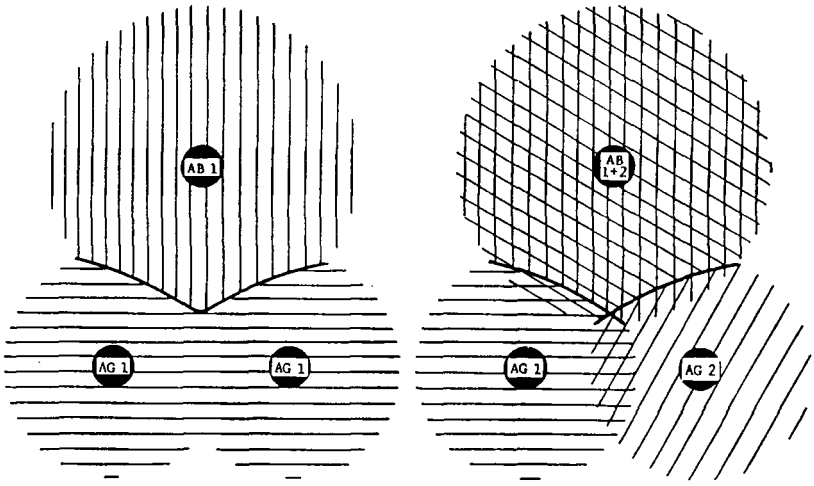

Figure 1 Diagrams of the fusing (left) and crossing (right) of precipitate lines in double-immunodiffusion. (Left) When two identical Ags (AG1), deposited in two different wells, can both precipitate with an Ab (AB1) from the third well, fusing must occur because of the specific impermeability of the precipitate line formed. (Right) When two different Ags (AG1 and AG2), deposited in two separate wells, each precipitates with its own Ab only (AB1 and AB2, respectively, mixed together in the third well, on top), the precipitate lines cross, as each is part of an entirely different system, in which each forms its own self-repairing, specifically impermeable precipitate barrier, which has nothing in common with the other.

given Ag–Ab system persists only as long as equivalent amounts of Ag *and* of Ab remain present in solution, each on its own side of that barrier. This requirement furnishes the explanation for the specific impermeability, which is based on the fact that the barrier is *self-repairing*; that is, as soon as a hole is formed in it by accident, some of the soluble Ag present on one side of it will penetrate that hole, but then immediately will encounter an equivalent amount of the soluble Ab present on the other side of the barrier, and forms a precipitate with it, which plugs the hole (van Oss and Heck, 1961).

2. Place of First Formation of Precipitate Lines

To understand properly the behavior, formation, evolution, and decay of Ag–Ab precipitate lines formed by double-diffusion in gels, it is indispensable to treat the first formation of precipitate lines as an entirely separate phenomenon from their later evolution, subsequent to their initial formation.

The Ag–Ab precipitate-forming systems, being essentially nonstoichiometric (see above), are *complex-forming systems* (as are precipitates or complex coacervates formed; e.g., by the interaction of anionic with cationic surfactants). Such systems tend to start precipitating when their concentrations are at equivalence (E) and have reached a certain minimum value, which is contrary to the precipitation of most non–complex-forming systems, which occur once a minimum is exceeded in the guise of the solubility product. It can be proven fairly easily that because Ag–Ab precipitation occurs when

$$C_{Ag} = C_{Ab} = E \tag{3}$$

the *place* where that precipitate first starts in double-diffusion is largely independent of the starting amounts deposited in the respective wells (van Oss, 1968; 1984). The place where the Ag–Ab precipitate first forms depends only on the distance ($a + b$) between the wells (see Fig. 2) and on the diffusion coefficients of Ag and Ab, such that

$$\frac{a}{b} = \sqrt{\frac{D_{Ag}}{D_{Ab}}} \tag{4}$$

As every point on the precipitate line of first formation has to obey that rule [Eq. (4)], the line of first formation, when the starting points are point-shaped (or small cylindrical) wells, must have the shape of a *circle*, with

$$R = \frac{ab}{a - b} \tag{5}$$

(Fig. 2). When $D_{Ag} = D_{Ab}$ (which occurs, for example, in IgG–anti-IgG systems), $a = b$ and $R = \infty$, resulting in a straight line perpendicular to the line connecting the Ag and Ab wells, and equidistant to these wells.

A point-shaped well versus a linear trough yields parabola-shaped precipitate lines, and two linear troughs give rise to linear precipitate lines (see later Sec. I.F.4).

Equation (4) can be used for the determination of the diffusion coefficient of a given Ag, if the diffusion coefficient of the corresponding Ab (or vice versa) is known. The best procedure for such a determination is shown in Fig. 3, in which two perpendicular rectilinear troughs are filled with Ag and Ab, respectively. The precipitate line obtained forms an angle such that

$$\tan\alpha = \sqrt{\frac{D_{Ag}}{D_{Ab}}} \tag{4A}$$

(Allison and Humphrey, 1959). By this procedure one has the unique possibility of determining the diffusion coefficient of an Ag without actually purifying it, provided that one has some means (e.g., by an enzymatic color reaction, or by an immunological reaction of identity) to identify the line formed by the Ag. In the application of this method, it is useful to know that for IgG, $D_{IgG} \approx 4 \times 10^{-7}$ cm^2/s, at 20°C.

3. Further Evolution of Precipitate Lines

If a precipitate line is formed when Ag and Ab are present at equivalent concentrations in their respective wells, the precipitate lines they form will indefinitely remain in the same place where it

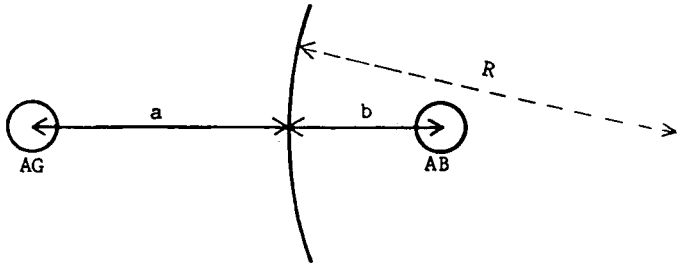

Figure 2 Circle-shaped precipitate line obtained by double-diffusion precipitation of an Ag with an Ab, such that $\sqrt{D_{Ag}}/\sqrt{D_{Ab}} = a/b$. The radius of the circle $R = ab/(a-b)$ [see Eq. (5)].

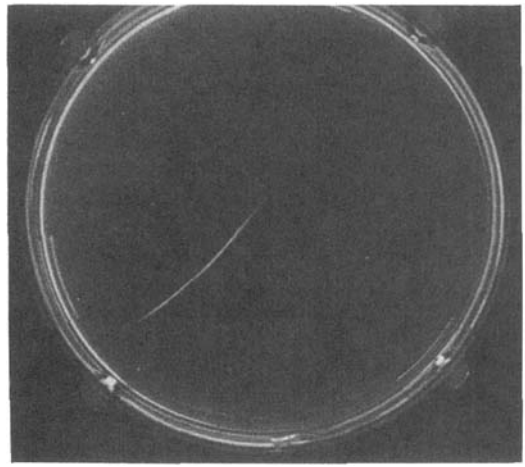

Figure 3 Two perpendicular troughs filled with, respectively, Ag (bovine serum albumin; BSA) horizontally and Ab (goat anti-BSA antiserum) vertically. The angle α the precipitate line makes with the horizontal trough is characterized by $\tan \alpha = \sqrt{D_{Ag}/D_{Ab}}$ [see Eq. (4A)]. For an Ab of the IgG class, the diffusion coefficient, $D_{Ab} = 4 \times 10^{-7}$ cm^2 s. From D_{Ab} and $\tan \alpha$, D_{Ag} can thus be derived from Eq. (4A). In this case, for $\tan \alpha \approx 1.22$, $D_{Ag} \approx 6 \times 10^{-7}$ cm^2/s, which corresponds to the diffusion coefficient of BSA.

was first formed, and it will remain thin. However, if one of the reagents (A) was present in its starting well in excess, it will ultimately be able to break down the barrier (when the other reagent begins to be exhausted), cross it, and form a new one farther down, in the direction of the reagent present in the lower concentration (B), with which it will react in a place closer to the starting well of B, where some B is still present in solution, etc. Thus the precipitate may do one of three things (van Oss and Heck, 1961):

1. It can *thicken* in the direction of the reagent (B) initially present in the lowest concentration.
2. It can form new precipitate lines in the direction of the reagent (B) initially present in the lowest concentration; instead of one thick line, *multiple lines* will form.
3. Excess reagent A may dissolve the line of first formation, and form a new precipitate line closer to B; the precipitate line will then appear to *move* in the direction of the reagent (B) present in the lowest concentration.

The visible evolution of the place or thickness of the precipitate line when one of the reagents is present in excess, in contrast with the line's continuing immobility and thinness when Ag and Ab are present at equivalent concentrations, makes it possible to do a simple determination of the equivalence ratio of a given Ag–Ab system by a double-diffusion titration (van Oss and Heck, 1961; van Oss, 1984a; Ouchterlony, 1968, p. 32) (Fig. 4).

4. Immunoelectrophoresis

As is often the case with two different methods, applied successively and in directions perpendicular to each other, the resolution of immunoelectrophoresis is a decimal order of magnitude greater than that of electrophoresis alone. Immunoelectrophoresis consists of the electrophoretic separation of an antigen mixture in the first dimension, followed by the characterization of each

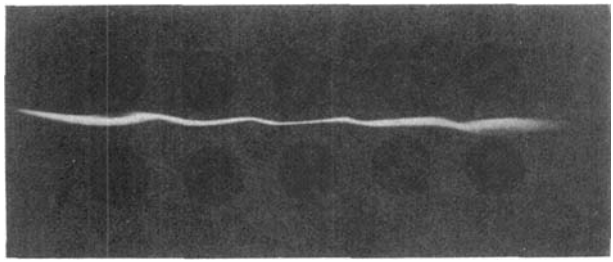

Figure 4 Titration of bovine serum albumin (BSA) with goat anti-BSA antiserum. (Top wells) Goat anti-BSA (diluted 1/2); bottom wells (left to right) 0.5%, 0.25%, 0.125%, 0.0625%, and 0.0312% BSA. The equivalence point (point of optimal Ag/Ab ratio) is at 0.125% BSA.

antigen by double-diffusion precipitation in a direction perpendicular to the electrophoretic step (Grabar and Burtin, 1964; Ouchterlony, 1968). The property of nonidentical antigens to form crossing precipitate lines on interaction with their corresponding antibodies (even when the antigens still partly overlap after a less than total electrophoretic separation) allows the characterization of overlapping antigens (Ouchterlony, 1968). The shape of the precipitate lines permits the identification of monoclonality (a parabola-shaped line, e.g., serum albumin) or polyclonality (a fairly straight line, e.g., IgG) of the separated fractions (see foregoing Sec. I.F.2). For this and other reasons, immunoelectrophoresis is especially useful for detecting monoclonal gammopathies. Because of an electrophoretic dilution effect (compounded by possible electrophoretic heterogeneities of given fractions), immunoelectrophoresis is less sensitive than ordinary double-diffusion immunoprecipitation tests, although with the help of radioactive Ag or Ab, by means of autoradiography, extremely small amounts of Ag or Ab can still be demonstrated by radioimmunoelectrophoresis.

5. Nondiffusion-Driven Antigen and Antibody Transport Mechanisms for Immunoprecipitation

Counterelectrophoresis (also referred to as crossed-over electrophoresis, electrosyneresis, immunoelectroosmophoresis, or immunoosmophoresis) brings Ag and Ab together by means of electrophoresis. This is possible only at a pH that is intermediate between the isoelectric pHs of Ag and Ab; in other words, Ag and Ab must have different isoelectric points for this method to be applicable. The method is faster (precipitate visible in a few hours) and more sensitive (by a factor of about 3 to 4) than double-diffusion precipitation. Contrary to the implication of some of the names proposed for this method, electroosmotic flow plays no role in bringing Ag and Ab together.

Rheophoresis, or immunorheophoresis, is a method for bringing Ag and Ab together by hydrodynamic transport engendered by the evaporation of solvent through a gap in the cover (on top of the gel chamber) situated exactly above the place (between Ag and Ab) in the gel where the precipitate is to occur. Excess solvent should be provided at both extremities of the gel, to furnish liquid for capillary transport through the gel's pores. This method is also relatively fast (precipitate visible in 2–4 h) and more sensitive (by a factor of 3 to 4) than double-diffusion, and can even be used when Ag and Ab have close or identical isoelectric points (van Oss and Bronson, 1969).

G. Single-Migration Precipitation in Gels

1. Radial Immunodiffusion

In single-migration methods, only one of the components (usually the Ag) migrates or diffuses into a gel that already contains a homogeneous, low concentration of the other component (usually the Ab). The simplest example of this type of method is single-diffusion, or radial immunodiffusion (Mancini et al., 1965), in which various concentrations of an Ag solution are deposited in wells punched in an Ag-containing gel. The initial concentrations of Ag deposited in the wells must be higher than the Ab concentration of the bulk of the gel for any Ag–Ab precipitate to become visible beyond the confines of the wells (see earlier Sec. I.F.3). After the precipitate ring around a well has reached its maximum size, the surface areas of the rings (or their diameter squared) are proportional to the amount (and thus to the concentration) of the Ag deposited in that well. Plots of Ag concentration versus precipitate ring diameter squared therefore yield straight lines. This method represents a reliable, but slow (24–48 h), method for measuring the concentration of plasma proteins and other Ags. Other methods of plotting Ag concentrations versus precipitate ring size are incorrect and usually inaccurate.

2. Electrophoretic Migration Methods in Antibody-Containing Gels

Instead of waiting for the diffusion of an Ag into an Ab-containing gel to run its (slow) course, the process can be accelerated by electrophoresing the Ag into the Ab-containing gel until all growth of the precipitate lines (now rocket-shaped) has stopped (Laurell, 1965). The final surface areas of the rockets (or their heights, as in first approximation the rockets may be likened to triangles with a unit baseline) are proportional to the Ag concentration. This method, which is somewhat more accurate than radial immunodiffusion (see above), is also about a decimal order of magnitude faster.

Bidimensional electrophoresis in Ab-containing gels (or crossed-immunoelectrophoresis) is comparable with immunoelectrophoresis in which the double immunodiffusion step has been replaced by electrophoresis (at right angles to the first electrophoresis step) into an antiserum-containing gel. With complex Ag mixtures, this gives rise to a forest of rockets; the surface area under each of these is roughly proportional to the concentration of the corresponding Ag originally present in the mixture (see, e.g., van Oss, 1984b).

3. Affinity Electrophoresis

Affinity electrophoresis uses the degree of retardation of the electrophoretic mobility of an Ag in a gel column in which the Ab is bound (or admixed with) the gel for obtaining equilibrium constants (see Chap. 23). Various ways of monitoring the rate of advance of Ag in the gel column can be used, including the rate of advance of an Ag–Ab precipitate, when such a precipitate is formed. However, as the degree of retardation of the Ag–Ab precipitate does not necessarily accurately reflect the degree of retardation of the electrophoretic mobility of the bulk of the Ag (under precipitating as well as nonprecipitating conditions), affinity electrophoresis, when measured by the progression of the Ag–Ab precipitate front, contains the risk of yielding erroneous equilibrium constants (van Oss et al., 1982a; see below).

4. Affinity Diffusion

If one can use affinity electrophoresis to measure the equilibrium constants of Ag–Ab interaction, one may also use affinity *diffusion* for the same purpose, in which case one measures the decrease in the diffusion coefficient of the Ag, in the Ab-containing gel, as a function of Ab concentration (van Oss et al., 1982b). However, as in affinity electrophoresis (see above), if the *progression of the Ag–Ab precipitate* front is measured for the purpose of deriving the dissociation constant of

the Ag–Ab interaction, there is a serious risk of obtaining erroneous results. The reason for this is that in this manner one tends to measure the dissociation constant of exactly those precipitate-forming reagents with the strongest propensity to dissociation (van Oss et al., 1982a).

Oudin noted in 1949 (Oudin, 1971) that, as in molecular diffusion, the rate of progression of Ag–Ab precipitate fronts by single diffusion in Ab-containing gel tubes (with a supply of excess Ag in solution on top of the gel tubes) is proportional to the square root of time. However, it was not realized until recently that the rate of progression of such Ag–Ab precipitate fronts is considerably faster than the rate corresponding to the diffusion coefficient of the Ag in free solution (van Oss et al., 1982a; see also the data furnished by Oudin, 1971). The explanation of this apparent paradox is relatively simple: for example, the molecular diffusion coefficient of albumin, as measured by visible or ultraviolet light absorption, is obtained from the progression of the inflection point of the first derivative of the absorption versus distance curve with time. However, the progression of albumin in an anti-albumin-containing gel, as judged by the *advancing precipitate front*, reflects only the diffusivity of the fastest few Ag molecules that can just attain the concentration necessary to achieve precipitation. These few fast Ag molecules tend to be many times faster than the molecules diffusing with the average of the bulk of the Ag (van Oss et al., 1982a).

5. Dissociation Constant Obtained by Affinity Methods

Both affinity electrophoresis and affinity diffusion (van Oss et al., 1982b), depend on the degree to which, for example, Ag, when migrating into an Ab-containing gel, slows down as a function of Ab concentration. The *dissociation* constant K_d can be obtained according to

$$\frac{D_0}{D_i} = 1 + \frac{C_i}{K_d} \tag{6}$$

where D_0 is the electrophoretic mobility (or diffusion coefficient) of Ag in the gel without Ab, and D_i the electrophoretic mobility (or diffusion coefficient) of Ag in the gel at Ab concentration C_i. When D_0/D_i is plotted on the ordinate versus C_i on the abscissa, one finds $-K_d$ at the intercept of the (straight) line of the function with the abscissa, or $1/K_d$ as the slope of the line. The zero Ab concentration diffusion coefficient (D_0) of Ag is found by extrapolation to zero Ab concentration (van Oss et al., 1982b). One can obtain K_d [Eq. (5)] only in ideal (totally homogeneous) systems, for Ags with only one specificity of antigenic determinant, or with monoclonal Abs (in which case one would not expect precipitation to occur). However, with heterogeneous Ag–Ab systems, one finds K_d values (at least with affinity electrophoresis and diffusion methods visualized by an advancing precipitation front) that are several decimal orders of magnitude higher than those found by precipitation in tubes, as one tends mainly to measure the components with the highest K_d value (see above). A further reason for the different values found with affinity and equilibrium systems lies in the fact that affinity methods are kinetic systems, for which one does not wait for complete equilibrium to set in, so that secondary interactions may not have had time enough to occur. Curiously enough, however, at least in the BSA–anti-BSA system, affinity diffusion results mainly seem to reflect "hydrophobic" interactions; they occur even at pH 9.5, at which electrostatic interactions in this system are largely inoperative and where precipitation does not take place in liquid aqueous solution, in the absence of a gel (van Oss et al., 1982a).

II. AGGLUTINATION

A. Introduction

Agglutination, one of the oldest methods for demonstrating the occurrence of immune reactions in vitro, is the destabilization of relatively stable suspensions of Ag particles by cross-linking

them with Abs directed to their Ag determinants (epitopes). Because destabilization of Ag particles can readily be detected with rather small volumes (ca. 0.1 ml) of dilute particle suspensions and because relatively few Ab molecules suffice to achieve destabilization, agglutination is an uncommonly sensitive method for detecting quite low concentrations of Ab (as low as a few nanograms per milliliter). The sizes of Ag particles, used in agglutination, may range from a few nm to about 10 μm in diameter. Antigenic particles may be cells (bacteria, red blood cells, or other) displaying their native epitopes, or inert particles (cells as well as synthetic carriers) to which Ag molecules have been adsorbed or covalently attached.

B. Visualization of Agglutination

1. Sedimentation Rate

According to Stokes' law, the force resisting sedimentation of a particle in a liquid medium is proportional to its radius, while the force inducing its sedimentation is proportional to the radius cubed. The net force causing a particle's sedimentation thus is proportional to the square of its radius. Therefore (other parameters being equal), when clumping causes an increase in particle size by a factor X, the clumps will sediment X^2 times faster than the initial monodispersed particles. For instance, single human erythrocytes, suspended in saline water, sediment at a rate of about 1 cm/h at ambient gravity, while clumps of agglutinated erythrocytes, comprising an average of 30 cross-linked cells, sediment 1 cm in about 6 min under the same conditions. Thus, the simple visual observation of a tenfold increase in sedimentation rate of a red cell suspension in a test tube is indicative of significant agglutination (van Oss, 1984b). The same holds true for accelerated sedimentation in a centrifugal yield, but as most cells (especially large cells such as erythrocytes) sediment rather quickly, even at relatively low forces of gravity (g), the use of centrifugation in assessing agglutination focuses more on the scrutiny of the physical properties of the completely sedimented packed agglutinate than on the actual sedimentation rate (which is difficult to measure under conventional circumstances).

2. Properties of Sedimented Agglutinates

Once cells (agglutinated or otherwise) have been sedimented by centrifugation, there are two ways of recognizing agglutination: (1) by the difference in adherence to the rounded bottom of the test tube, and (2) by the difference in dispersability that may be observed while attempting to resuspend the sedimented cells (e.g., by shaking).

Nonagglutinated, monodispersed cells or particles pack very tightly when forced to the bottom of a test tube by a centrifugal field; nonagglutinated cells are therefore deposited in a small, round, sharply delineated button at the bottom of the tube. Agglutinated cells, on the other hand, form large, open network structures of many cells attached to each other at a few points only; such large "flocs" cannot be packed tightly and thus tend to be deposited on, and adhere to, the entire hemispherical inner surface of the bottom of the tube. Consequently, sedimented agglutinates occur as wide and thinly spread layers, often with jagged edges, covering much of the surface of the bottom of the tube, in sharp contrast with the small, round buttons formed by nonagglutinated cells.

For further verification, or when too few cells are available for visual inspection of the deposited cells with the naked eye, resuspension of the cells may be attempted (e.g., by vigorous shaking of the test tubes). Nonagglutinated cells can be completely redispersed in this manner, while agglutinates are indispersable and remain present as large flocs. With large amounts of cells, that phenomenon is visible with the naked eye; however, when relatively few cells are present in each tube, one should revert to microscopic inspection of the contents of each tube.

3. Agglutination on Flat Surfaces

Agglutination can also be performed on flat plates (made of glass, plastic, or even cardboard). By that technique agglutination is recognized visually by the coarse graininess of agglutinated cell clumps, in contrast with the smooth aspect of monodisperse cell suspensions.

4. Agglutination in Gels

Agglutination of very small particles (e.g., sonicated fragments of erythrocyte stromata) can even be effected in gels, by double-diffusion of such very small antigenic particles (Milgrom and Loza, 1967).

5. Automated Agglutination Methods

To quantitate the results of many hemagglutination determinations in quick sequence, the agglutinated cells can be separated from the nonagglutinated cell suspensions; the latter may be hemolyzed and the hemolysate conducted through a spectrophotometer which can record, *via* the absorptivity at 420-nm wavelength, the proportion of nonagglutinated cells, thus expressing, by difference from the total amount of cells used, a measure of the number of cells that have been agglutinated; for example, as a function of the concentration of Ab to be tested (Greenwalt and Steane, 1973).

For the quantitation of Ab (or Ag) by passive latex agglutination, the number of nonagglutinated particles (which are all close to the same size) can be automatically determined by light scattering at such (relatively small) forward light-scattering angles as will best assure the exclusive counting of small particles that are of the size of nonagglutinated particles only (Masson et al., 1981).

C. Hemagglutination

1. Hemagglutination with Immunoglobulin M and Immunoglobulin G Class Antibodies

From the early days of the clinical application of blood transfusion, hemagglutination with blood group Abs has been the principal analytical tool in bloodbanking and immunohematology. It has long been known that with "complete" IgM class Abs, due to their size as well as to the availability of ten Ab sites (Edberg et al., 1972), disposed at diametrical distances of about 30 nm, hemagglutination is much more readily achieved than with "incomplete" Abs of the IgG class, which have only two Ab sites which are maximally about 12–14 nm apart (van Oss, 1984b; 1989; 1990).

However, as incomplete IgG class Abs are also of considerable importance among blood group Abs, much effort has been devoted to modifications of the environment and properties of erythrocytes to facilitate hemagglutination with IgG. With some IgG class blood group Abs (e.g., anti-A and anti-B) on the other hand, hemagglutination is easily achieved; the reasons for this are discussed below.

2. Distance Between Cell Surfaces

When the surface tension components and the electrokinetic surface (ζ)-potential of a cell type are known, it is possible to construct a plot of the free energy of attraction (or repulsion) (ΔG_{iwi}^{TOT}) between two interacting cells, i, immersed in an aqueous medium, w, as a function of the distance (ℓ) between the outer edges of two such cells (van Oss, 1989; 1990). Figure 5 shows a ΔG_{iwi}^{TOT} vs ℓ graph for human erythrocytes in the flat–flat (i.e., rouleau) formation as well as in the edge–edge conformation. The intercellular distances, ℓ, which are depicted in Fig. 5, are counted from the distal edges of the cellular glycocalices.

Under physiological conditions of pH and ionic strength, the outer edges of the glycocalices

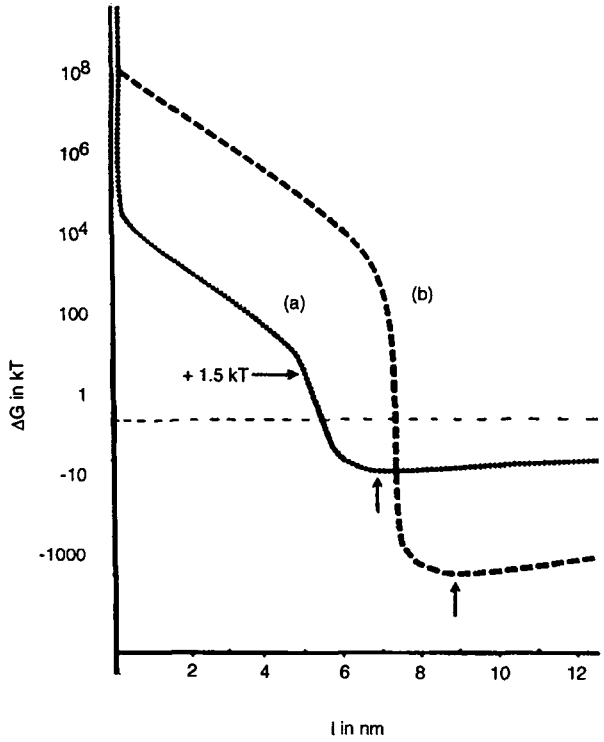

Figure 5 Energy balance of human erythrocytes, taking ΔG^{EL}, ΔG^{LW}, *and* ΔG^{AB} into account. The decay length of water is taken to be $\lambda = 0.6$ nm (see Chapter 23, and text). The left-hand curve (a) is computed by assuming a radius of curvature at the approach of two cells of $R = 1.5$ μm. The right-hand curve (b) is based on the interaction between two cells in the flat parallel slab mode, with a surface area of approach $S_c = 25.9$ μm^2. The vertical arrows indicate the secondary minima of attraction of the two modes a and b. A horizontal arrow indicates the place on curve (a) of $\Delta G = +1.5$ kT, corresponding to $\ell \approx 5.0$ nm, which is the most likely minimum distance between glycocalyx surfaces of two red cells approaching each other by their convex edges, under the influence of their Brownian motion. The relative slight elastic repulsion engendered by the cell encounters at $\Delta G = +1.5$ kT is not taken into account here. (From van Oss, 1990.)

of erythrocytes cannot approach each other to an intercellular distance, ℓ, smaller than about 4–5 nm (van Oss, 1989; 1990), which, however, makes the distance at the secondary minimum of attraction (see Fig. 5) between the actual cell membranes of two opposing erythrocytes about 15–16 nm, which is slightly more than the "reach" of IgG class Abs (12–14 nm), but quite sufficient for cross-linking by IgM class Abs (27–28 nm) (Fig. 6). The interactions which force erythrocytes to keep a certain distance apart are threefold: a Lifshitz–van der Waals (LW) attraction ($\Delta G^{LW}_{\ell_0} \approx -0.6$ mJ/m^2); a hydrogen-bonding (acid–base; AB) repulsion ($\Delta G^{AB}_{\ell_0} = +25$ mJ/m^2); and an electrostatic (EL) repulsion ($\Delta G^{EL}_{\ell_0} = +0.5$ mJ/m^2). Each of these interactions follow a different regimen in their decay as a function of distance, resulting in a secondary minimum of attraction at a distance between the distal edges of the glycocalices of

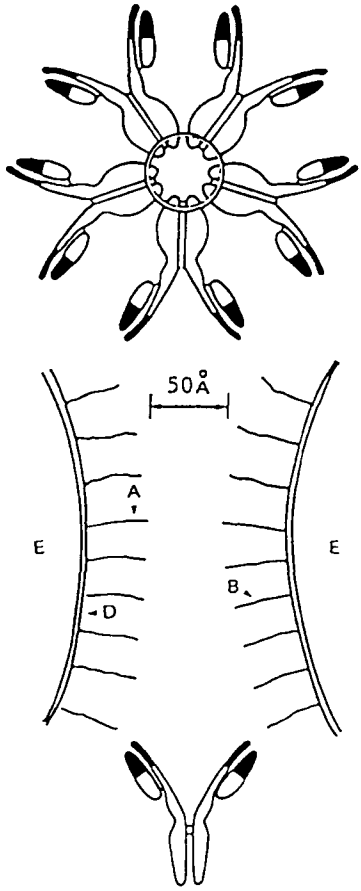

Figure 6 Diagram of the minimum distance of approach of two normal human erythrocytes, with an IgG and an IgM molecule drawn on the same scale. The curvature of the erythrocyte surfaces has been drawn vastly exaggerated, considering the scale: on this scale the diameter of an erythrocyte would be about 33 ft (≈10 m). E indicates the interior of the erythrocytes; A, B, and D indicate sites of A, B, and D (Rh_o) antigenic determinants. The closest approach between two unsensitized erythrocytes is ≈50 Å from each cell membrane's lipid bilayer.

about 5 nm in the edge–edge conformation (see Fig. 5). As can be seen in Fig. 6, IgM class Abs can always cross-link erythrocytes, whether the epitopes are situated on the glycocalyx strands (which are never less than about 5 nm apart), or on the cell membranes (which are at least 15 nm apart). IgG class Abs, however, can cross-link erythrocytes only when the epitopes are situated on the glycocalyx edges (which occurs with ABO blood group Ags), but not when the epitopes are part of the cell membrane (which is the case with Rh blood group Ags). Therefore, to facilitate hemagglutination with anti-Rh Abs (which tend to be of the IgG class), one of the two types of

measures can be taken: (*1*) the cells can be pushed closer together, or (2) the reach of IgG class Abs can be extended.

3. Methods for Increasing the Reach of Immunoglobulin G Class Antibodies

Chemical Approach. The maximum distance between the two paratopes on one IgG molecule can be increased by mild reduction followed by alkylation (Romans et al., 1977), which results in breaking at least some of the inter-heavy chain disulfide bonds in the hinge region. Not all inter-heavy chain disulfide bonds should be broken because that would result in monovalent Ab pieces, which are incapable of cross-binding. A "reach" of about 15–16 nm must be attained to achieve hemagglutination. Therefore, it seems probable that this method mainly applies to the opening up of IgG3 with its eleven S—S bonds and, to a lesser extent, to IgG2 with four S—S bonds (Burton et al., 1986).

Immunochemical Approach. The most important method of increasing the "reach" of IgG class Abs is the *indirect* antiglobulin, or Coombs test (Coombs et al., 1945). By this approach, two or more red cells monogamously sensitized with IgG class Abs are subsequently cross-linked by means of (usually rabbit) antihuman IgG Abs. A schematic illustration of this mode of cross-linking is given in Fig. 7. It is a two-step approach, but the end result is that cross-binding occurs through two human IgG molecules (one at each end), each about 12–14 nm long, which

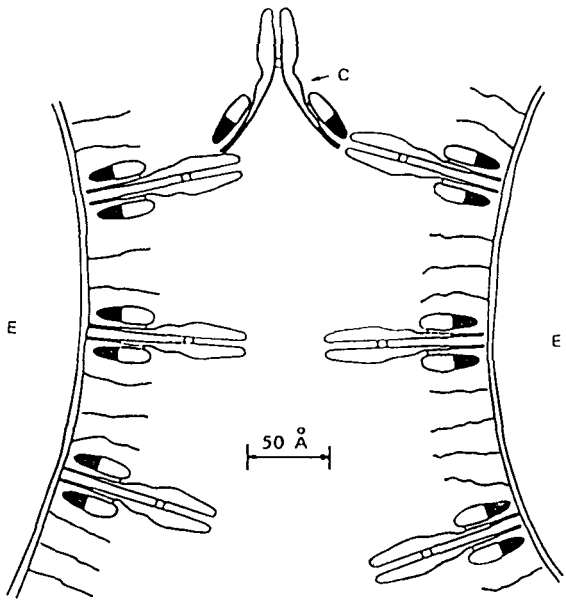

Figure 7 Diagram of the minimum distance of approach of two human erythrocytes, E, monogamously sensitized with IgG class anti-D(Rh_o) Abs. The curvature of the erythrocyte surfaces has been drawn vastly exaggerated, considering the scale. The closest approach between sensitized cells is ≈40 Å (from Fc tail to Fc tail). The extremities of two opposing IgG Fc tails obviously can easily be cross-linked by another (rabbit–antihuman IgG, or "Coombs") IgG class molecule, here indicated by C.

are linked together by one (rabbit) antihuman IgG molecule with a "reach" of about 12 nm, resulting in an extended tetravalent Ab, with a total "reach" of ≈36 nm (i.e., slightly larger than that of IgM). For all cross-matching tests used for blood transfusions and for other tests for anti-Rh Abs, the use of the indirect antiglobulin or Coombs test remains indispensable.

The *direct* antiglobulin test (Coombs et al., 1946) (i.e., the testing for erythrocytes already sensitized in vivo, by means of antihuman IgG Abs) is used for the detection of sensitization in cases of hemolytic disease of the newborn.

4. Methods for Reducing the Intercellular Distance

Decreasing ℓ by Centrifugation. Centrifugation can be used to bring $D(Rh_o)$-positive erythrocytes close enough together for cross-linking with incomplete IgG class Abs at 15,000 *g*, but not at 3,750 *g* (Hirszfeld and Dubiski, 1956; van Oss, 1985).

Decreasing ℓ by Reduction of the Intercellular Repulsion. Reduction of the intercellular repulsion is mainly effective when especially directed to the polar, hydrogen-bonding (AB) repulsion, as the *polar repulsion* between red cells under physiological conditions accounts for 98% of the total intercellular repulsive forces (see above). The electrostatic repulsion amounts to only 2% of the total repulsive forces, and the Lifshitz–van der Waals attraction also is only about 2% of the total interaction (all at closest approach).

The polar intercellular repulsion can be decreased by most methods which also cause a decrease in the cells' surface (ζ) potential. Whilst treatment of red cells with neuraminidase, bromelin, or papain, as well as with plurivalent cations, causes a significant decrease in their ζ-potential, the more important effect of enzymatic or cation treatment of erythrocytes is that they also become *less hydrophilic*, thereby decreasing their polar (AB) repulsion, which then results in a decrease in their intercellular distance, ℓ (van Oss and Absolom, 1984; van Oss, 1994).

Papain is among the most effective enzymes in decreasing the intercellular distance; it is widely used in facilitating hemagglutination with ("incomplete") IgG class Abs. Among salts with multivalent cations, those with the highest valency are the most effective in destabilizing negatively charged particles, including erythrocytes (van Oss, 1993; 1994). Tannic acid treatment (which also renders erythrocytes more "hydrophobic") also is effective in bringing red cells closer together; this treatment also enhances hemagglutination with "incomplete" IgG class Abs (Pirofsky et al., 1962).

Decreasing ℓ by Exertion of Extracellular Pressure. Extracellular pressure is the mechanism by which one facilitates hemagglutination by IgG class Abs through the admixture of relatively high concentrations of serum albumin, dextran, polyvinyl pyrrolidone, and other water-soluble polymers (van Oss, 1993). When a sufficiently high concentration of water-soluble polymer is reached (e.g., of the order of at least 2–10%), a phase separation occurs between the cells and the polymers (the polymer molecules themselves may partly adsorb onto the cells, but that does not alter the mechanism of the phenomenon). When the polymer concentration becomes high enough, the polar (AB) free energy of repulsion of the polymers becomes higher than that of the cells. This reduces the intercellular distance, ℓ. With serum albumin, concentrations of 12–20% (w/v) are used for that purpose while, typically, dextran concentrations between 5 and 10% suffice to decrease ℓ.

Decreasing ℓ by Cross-Linking the Cells with Asymmetrical Polymers. This approach is based on a totally different mechanism from the one discussed in the previous section. *Cross-linking with polymers* occurs at relatively low polymer concentrations and is done with (1) very asymmetrical, high molecular weight neutral or negatively charged polymers which cross-link through adsorption or entanglement with two (or more) cells per polymer molecule; or (2) already

adsorbed biopolymer molecules which are made insoluble; or (3) positively charged polymers. Cross-linking with the first type of polymers gives rise to the rouleau-formation type of hemagglutination. Red cells *preferentially* attract each other in the parallel disk conformation at an intercellular distance, ℓ, of about 7 nm. Figure 5 shows that the energy of attraction of red cells at the secondary minimum is about three orders of magnitude greater in the parallel disk configuration than in the edge–edge configuration. Red cells thus have a natural propensity to form rouleaux. However, in the normal course of events, red cells will not actually form stable rouleaux because even minute hydrodynamic disturbances will cause the cells to slide away from each other. It should be remembered that the maximum of attraction occurs when two parallel biconcave disks still are about 7 nm apart: when they approach one another only slightly more closely, the attraction turns into an even greater repulsion. Thus, stable rouleau formation occurs only when the energetically favored parallel disk situation can become stabilized in that conformation through the anchoring action of cross-linking string-like polymer molecules (van Oss and Absolom, 1985). This sort of erythrocyte aggregation can be achieved with a variety of asymmetrical polymers: fibrinogen, dextran, nucleic acids, heparin, and polymerized albumin (van Oss, 1993). The higher the molecular weight of an asymmetrical polymer, the lower the concentration needed to obtain rouleau formation: with dextran Mw $\approx$ 100,000, 1% causes rouleau formation, while with dextran Mw $\approx$ 270,000, a 0.4% concentration of the polymer suffices (Mollison, 1972). The cross-linking induced by dextran can be inhibited by glucose (van Oss et al., 1978), which suggests that the strong adsorption of dextran onto the erythrocyte glycocalyx may be due to the presence of a lectin-like peptide on the cell surface with glucose specificity.

Cross-linking with a second class of polymers has an entirely different mechanism. It is mediated by the normally occurring aspecific adsorption of *euglobulins* (mainly of the IgM type) from the serum onto the red cells, which normally occurs under physiological conditions, but which does not, by itself, cause cross-linking. However, when the ionic strength of the suspending medium is lowered, the adsorbed euglobulins precipitate and, in so doing, interact with each other intercellularly, causing cross-linking. When red cells are washed to remove the adsorbed proteins, this type of cross-binding is no longer possible, but when the eluted euglobulins are added back into the red cell suspension, cross-linking at low ionic strength resumes (van Oss and Buenting, 1967). The aspect of red cells agglomerated in this matter is different from that of regular rouleaux; some remnants of cell stacks can still be discerned, but all clumps look as though they were painted over (with layers of precipitated euglobulin), as observed by scanning electron microscopy (van Oss and Mohn, 1970). Addition of salt to cells agglomerated in this manner redissolves the euglobulins and allows the cells to redisperse.

Cross-linking of red cells with a third class of polymers can occur with *positively charged polyelectrolytes* (usually of relatively low to medium molecular weight), which cross-link erythrocytes by direct combination with (and neutralization of) the acid site of the glycocalyx. Complexes of this type are most easily formed at relatively low ionic strengths and redisperse on the addition of salt. Basic polymers of this type are polybrene [poly(hexadimethrine bromide)], protamine, and polylysine (van Oss, 1993). Neutralization of negatively charged sites on the glycocalyx also makes the cell surfaces more "hydrophobic" and, thereby, even more prone to agglutination.

Decreasing ℓ by Red Cell Spiculation. Cell surface processes in the form of spicules or spikes, with a small radius of curvature, undergo a much smaller repulsion than smooth parts of the cell surface. Spicules, therefore, can approach other cell surfaces much more closely than totally smooth cells. Anti-A as well as anti-B blood group Abs (as well as anti-A lectins) tend to cause spiculation in red cells, but anti-Rh_0 (D) Abs do not (Rebuck, 1953; Salsbury and Clarke,

1967; van Oss and Mohn, 1970). Thus, hemagglutination with anti-A or anti-B blood group Abs,even of the IgG class, never is a problem. However, major problems are encountered with anti-D(Rh_0) Abs, which usually are of the IgG class, and which leave the cells quite smooth. It should be noted that dextran (Mw $\approx$ 40,000) also causes spiculation (van Oss et al., 1978), which may be another reason for its usefulness in facilitating hemagglutination with "incomplete" IgG class anti-D(Rh_0) Abs. The reason why spiculation occurs with anti-A and anti-B Abs or lectins, and not with anti-D Abs, probably lies in the difference in the numbers of blood group A versus blood group D (Rh_0) epitopes per cell—the number of A epitopes being of the order of 10^6, while that of D epitopes is only of the order of 10^4 per cell (Masouredis, 1960; van Oss and Mohn, 1970).

D. Other Types of Agglutination

1. Inhibition of Hemagglutination

Inhibition of hemagglutination is used to measure the degree to which dissolved Ag can bind Ab, as shown by the inhibition of hemagglutination of cells coated with the same Ag. Inhibition of hemagglutination can also be used to characterize Abs to viruses that can agglutinate various species of erythrocytes (e.g., Abs to measles, rubella, influenza viruses).

2. Bacterial Agglutination

Among other agglutination methods, one of the oldest is bacterial agglutination, used for the demonstration of the presence of antibacterial antibodies in sera.

3. Passive Agglutination

To this point, mainly active hemagglutination has been discussed as used in blood grouping work. Hemagglutination can also be used as passive hemagglutination, with antigens that are not an integral part of the red cells, but may be adsorbed onto red cells that serve solely as carrier particles. Soluble polysaccharide Ags usually easily adsorb directly onto the red cell surface by interacting with lectinlike receptors on the erythrocytes. However, protein Ags will adsorb onto red cells only after the latter have undergone treatment with, for example, 0.005% tannic acid (10 min at 37°C) or 1.65% glutaraldehyde (several hours at room temperature). Protein Ags and haptens can also be covalently coupled to red cells by diazotization and other chemical attachment procedures, which can make passive hemagglutination and inhibition of (passive) hemagglutination one of the most sensitive methods for the characterization of small amounts of Ab (Adler and Adler, 1980); the method has proved especially useful for the characterization of monoclonal Abs. With erythrocytes coupled to protein antigens or haptens, one can of course also characterize Abs as well as Ab-forming cells by passive complement-mediated hemolysis (in tubes as well as in plaque assays) (Adler and Adler, 1980).

Passive agglutination with polystyrene latex and other particles has become an important tool in clinical tests, as well as in research, for the characterization of Ags as well as Abs, either of which can be aspecifically but firmly adsorbed onto the rather hydrophobic surfaces of latex particles or covalently bound to chemically modified lattices (see, e.g., Masson et al., 1981). The first passive latex agglutination test was developed by Singer (1961) for the demonstration of rheumatoid factor (IgM class Ab to slightly altered or aggregated IgG). In that method, human IgG is aspecifically adsorbed onto the surface of polystyrene latex particles (0.25 or 0.8 μm in diameter), which then can be agglutinated by sera that contain rheumatoid factor. Latex agglutination is also used to demonstrate the presence of chorionic gonadotropic hormone (usually as an inhibition of agglutination test) in pregnancy urine, and also, for example, in tests for C-reactive protein, antithyroglobulin, and antistreptolysin O Abs, as well as in the determination of levels of circulating immune complexes, IgE, α-fetoprotein, and so on (Masson et al., 1981).

4. Agglutination Involving Two Cell Types

Mixed agglutination is a technique usually applied to cultured cell monolayers for the characterization of Ags on the cultured cells, by the intermediary of Abs bound to these Ags. Erythrocytes, sensitized with antierythrocyte Abs, are used as indicators of the presence of sensitized cultured cells by being cross-linked to them with anti-immunoglobulin Abs, much like an antiglobulin or Coombs procedure (Coombs et al., 1945), arranged in two flat layers of different opposing cells, as in a sandwich.

Coagglutination is a procedure to characterize bacteria sensitized with type-specific Abs of the IgG class, by cross-linking them with *Staphylococcus aureus*, strain Cowan I, which carries protein A. *Via* protein A, which binds to the Fc moieties of IgG (in human IgG, to IgG1, IgG2, IgG4), the sensitized bacteria are then coagglutinated with formaldehyde and heat-treated staphylococci (Kronvall, 1973). When the type-specific Abs are not of the IgG class, coagglutination is not possible (Hovanec et al., 1980), but in the case of IgM, direct agglutination is practicable. Coagglutination is used for the characterization or typing of, for example, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, and various groups of streptococci. Coagglutination with protein A-bearing staphylococci also can be used to demonstrate sensitization with IgG class Abs of erythrocytes and other mammalian cells.

Closely related to coagglutination, as well as to mixed agglutination, are the various *rosetting* techniques. These have been developed primarily for the characterization of different kinds of lymphocytes. For instance, sheep erythrocytes (E) are prone to attach themselves to human T lymphocytes, and to arrange themselves into a circular formation around such lymphocytes, reminiscent of a flower. Such *rosettes* are not only useful for detecting human T cells, but also for removing T cells from lymphocyte preparations, making use of the fact that T lymphocytes rosetted with sheep erythrocytes (E rosettes) sediment much faster than the non–E-rosetted non-T lymphocytes. Table 1 summarizes a number of leukocyte-rosetting approaches. Protein A-bearing staphylococci, latex particles coated with IgG, and B lymphocytes have been used to characterize Rh(D)-positive erythrocytes sensitized with IgG class anti-Rh(D) Abs (EA; see Table 1) (Loren et al., 1982).

There is no significant qualitative or quantitative difference between rosetting and coagglutination, except that in rosetting one usually employs erythrocytes, while staphylococci are more typically used in coagglutination.

Table 1 Some of the Principal Modes of Leukocyte Rosetting with Erythrocytes (E)

Type of rosetting	Type of treatment of erythrocytes	Types of leukocytes rosetted
E	Sheep E	T cells; 50% of NK cells (low-affinity E rosettes)
EAγ	E, sensitized with IgG class antibodies	B cells, some T cells and NK cells; granulocytes, monocytes, macrophages
EAμ	E, sensitized with IgM-class antibodies	Some B and T cells
EAC	E, sensitized and treated with complement; ox erythrocytes are often preferred, to avoid binding T cells with nonsensitized patches (see sheep E, above)	B cells, some non-B cells

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