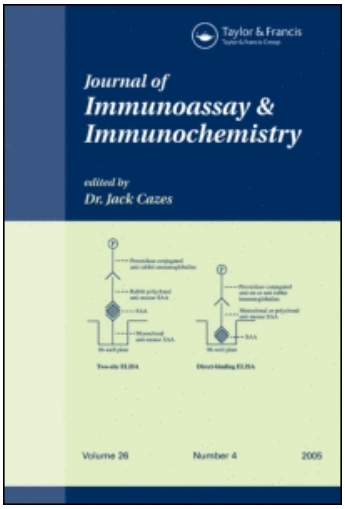


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# Nature of Specific Ligand-Receptor Bonds, in Particular the Antigen-Antibody Bond

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# Nature of Specific Ligand–Receptor Bonds, in Particular the Antigen–Antibody Bond

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## I. INTRODUCTION

The binding forces involved in the specific interactions between antigens (Ag) and antibodies (Ab), lectins and carbohydrates, ligands and their receptors and, in most cases, enzymes and their substrates, are of a noncovalent, purely physicochemical nature. The same (attractive) physicochemical forces that govern their binding also constitute the (repulsive) forces which prevent it [e.g., freely suspended peripheral blood cells to approach each other more closely than a given minimum distance (van Oss, 1989, 1990a)] and which allow proteins and other biopolymers to remain in aqueous solution (van Oss et al., 1986b). The three primary physical forces that play a role in Ag–Ab bonds are Lifshitz–van der Waals (LW), electrostatic (EL), and Lewis acid–base (AB), or electron-acceptor–electron-donor interactions. While it is usually possible to distinguish between LW, EL, and AB contributions to Ag–Ab bonding, for example, by observing the effect of changes in ionic strength (which influence EL, but not AB or LW forces), or of the admixture of polar organic solvents (which mainly influence AB forces), it remains difficult to discriminate between general AB interactions (which, in water, usually are mainly due to hydrogen-bonding), and *direct* hydrogen bond formation. It is important to be able to distinguish between the three primary forces. The difference in the rate of decay as a function of distance of the three primary physical forces involved in cell interactions as well as in Ag–Ab bonds, is a major reason for treating the three forces individually. The rate of decay with distance of the three forces involved in specific bonds plays an important role in the study of these interactions, because Ags and Abs and other ligands and receptors must be able to attract each other from a distance, which can be of the order of 3–8 nm. It should also be realized that various physicochemical changes one can make in the conditions under which Ags and Abs (and other receptors and ligands) interact (such as ionic strength, pH, temperature, content of organic solvent or other solute), can have a different influence on LW, or EL, or AB interaction forces.

For all symbols used, see list of Abbreviations, Symbols, and Units.

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Apart from the absence of covalent bonds, Ag–Ab interactions differ in other ways from more conventional chemical reactions. For instance, whilst the valencies of Abs are known precisely (they are either divalent or decavalent, dependent on the antibody class), the valencies of most Ags rarely are well defined. In addition, different valency sites (*epitopes*) of an Ag often (but not invariably) differ from each other in their chemical composition. Thus, plurivalency of most Ags usually only has meaning vis-à-vis polyclonal Abs, comprising many Ab populations, in which each population has a different antibody-active site (*paratope*). Even within only one population of (polyclonal) Abs that are all directed specifically toward one given epitope, the paratopes, while chemically fairly similar, still vary sufficiently in their amino acid composition to display a wide array of different affinities to that epitope.

Antigens can generally combine with their Abs in virtually any proportion, so that the concept of stoichiometry becomes irrelevant to Ag–Ab reactions. Even when the binding energy of an Ag–Ab reaction is determined at the optimal binding, or "stoichiometric" ratio, that binding energy is still strongly proportional to the concentration of the reagents (van Oss and Walker, 1986). This phenomenon greatly complicates the experimental conditions under which meaningful Ag–Ab-binding energies can be usefully determined, and seriously compromises the significance of many of the Ag–Ab-binding constants that have now been published. The interpretation of Ag–Ab-binding energies is further complicated by *hysteresis*; that is, the phenomenon whereby the energy of Ag–Ab *dissociation* is higher than the energy of *association*, due, for example, to the gradual formation of additional secondary Ag–Ab bonds of lesser specificity. The role of entropy in Ag–Ab reactions also is somewhat controversial. Although the formation of regular Ag–Ab lattices should reasonably be accompanied by an increase in order, in reality the formation of Ag–Ab complexes more often than not gives rise to a significant increase in entropy.



## II. THE ANTIGEN–ANTIBODY AND OTHER SPECIFIC BONDS

The Ag–Ab bond comprises the following three physicochemical forces: (1) Lifshitz–van der Waals (LW) or electrodynamic forces; (2) Coulombic or electrostatic (EL) forces*; and (3) electron-acceptor–electron-donor (Lewis acid–base) (AB)† or polar forces. Whilst in aqueous media LW forces are virtually always attractive, they are rarely quantitatively predominant; they usually represent less than 10% of the total interaction. The EL forces can be repulsive or attractive, dependent on whether they act on entities with the same, or with opposite signs of charge. Similarly, AB forces can be repulsive or attractive, dependent on whether they operate (in a polar medium) between hydrophilic or hydrophobic entities.

Ag–Ab bonds thus consist principally of electrostatic (EL) forces and polar (AB) forces in every conceivable proportion. Some types of Ag–Ab bond are predominantly of the EL variety (with a slight background of additional LW forces), others are purely of polar (AB) origin (with an equally minor LW background). And in a great many cases, Ag–Ab bonds comprise *combinations* of EL and AB forces (with in addition a small LW contribution). Especially when contemplating dissociation of Ag–Ab bonds, it becomes crucial to take the probability of a hybrid nature of such bonds into consideration, because the conditions favoring the dissociation of EL and AB bonds tend to differ significantly.

*Called "salt bridges" by some workers.

†Hydrogen bonds, in the guise of Brønsted acid–base interactions are a subset of Lewis acid–base interactions.

## III. LIFSHITZ–VAN DER WAALS FORCES

### A. Nature of Lifshitz–van der Waals Forces

There is a general attraction between all atoms and molecules that are brought closely enough together in vacuo, as well as in condensed-phase media, which is caused by the interaction between the fluctuating dipole, occurring in one atom, and a second dipole which the first dipole induces in a second atom. The resulting interatomic and intermolecular forces are called van der Waals–London, or dispersion forces. Of somewhat lesser importance in aqueous media, but by no means necessarily negligible, are two additional van der Waals interactions. These are (1) the interactions between permanent dipoles, called van der Waals–Keesom, or orientation forces; and (2) the interactions between a permanent dipole and a dipole induced by that permanent dipole, called van der Waals–Debye, or induction forces. However, following the Lifshitz approach, it can be shown that, on a macroscopic scale, van der Waals–Keesom and van der Waals–Debye interaction forces obey the same rules as van der Waals–London (dispersion) forces, in the condensed-phase state (Chaudhury,1984; van Oss et al., 1988; Good and Chaudhury, 1991). These forces are collectively alluded to as Lifshitz–van der Waals (LW) forces.

### B. Lifshitz–van der Waals Free Energies

The LW interactions between atoms or molecules are, to a significant degree, additive. Their free energy of interaction $\Delta G^{LW}$ (in the configuration of two semi-infinite flat parallel bodies) may be expressed as

$$\Delta G^{LW} = \frac{-A}{12\pi\ell^2} \tag{1}$$

where $A$ is the Hamaker coefficient (which is linked to physical properties of the interacting materials, including those of the liquid, when the interaction takes place in a liquid) and $\ell$ is the distance between the two parallel bodies or macromolecular sites. The LW interactions can be estimated by the Lifshitz approach (Visser, 1972) or, more easily, by determinations of the Lifshitz–van der Waals surface tension components (van Oss et al., 1988). The free energy $\Delta G^{LW}_{1W2}$ of the Lifshitz–van der Waals part of the interaction between two materials 1 and 2 (in an aqueous medium, $w$) can be determined as follows. For $\ell = \ell_0$ [$\ell_0$ is the minimum equilibrium distance between two parallel bodies or molecules; $\ell_0 \approx 1.57$ Å (van Oss et al., 1988)] $\Delta G^{LW}_{1W2}$ is expressed by means of the Dupré equation in condensed media:

$$\Delta G^{LW}_{1W2} = \gamma^{LW}_{12} - \gamma^{LW}_{1W} - \gamma^{LW}_{2W} \tag{2}$$

where $\gamma^{LW}_{ij}$ stands for the interfacial tensions between the materials indicated by the subscripts and where $\Delta G^{LW}_{1W2}$ is the same at $\Delta G^{LW}$ given in Eq. (1). Once the LW components of the surface components of the surface tensions of, for example, Ag (1), Ab (2), and of the liquid medium ($W$), $\gamma^{LW}_1$, $\gamma^{LW}_2$, and $\gamma^{LW}_W$, are known, the interfacial tensions of Eq. (2) can be obtained from the LW components of the surface tensions,* with an error of less than ± 2% (Good and Chaudhury, 1991):

$$\gamma^{LW}_{ij} = (\sqrt{\gamma^{LW}_i} - \sqrt{\gamma^{LW}_j})^2 \tag{3}$$

The apolar interaction between two similar molecules, or cells, 1, immersed in an aqueous liquid, $w$, can be expressed as:

$$\Delta G^{LW}_{1W1} = -2\gamma^{LW}_{1W} \tag{4}$$

*For methods of measurement of LW surface tension components, see van Oss (1994a); see also footnote, 5, below.

### C. Sign and Relative Importance of Lifshitz–van der Waals Forces in Antigen–Antibody Interactions

$\Delta G^{LW}_{1W2}$ can be negative (attractive) or positive (repulsive), depending on the value of the Hamaker coefficient $A_{WW}$ of the liquid medium, relative to the values of the Hamaker coefficients $A_{11}$ and $A_{22}$ of Ag and Ab. Thus, manipulation of some of the properties of the liquid medium, may turn an attractive reaction (association) into a repulsive reaction (dissociation) (van Oss et al., 1988). In *aqueous media*, however, which have a very low $\gamma^{LW}$ value ($\gamma^{LW}_W \approx 21.8$ mJ/m$^2$), the interaction between biological molecules or particles (usually with a $\gamma^{LW}_1$ value of the order of 40 mJ/m$^2$), $\Delta G^{LW}_{1W1}$ and $\Delta G^{LW}_{1W2}$ always have a negative value; that is, the LW interactions between biological entities, in aqueous media, virtually always are *attractive* with a maximum value of about $-5$ mj/m$^2$ between two *dry* biological entities (i.e., after expulsion of water of hydration). Between two *hydrated* biological entities, in water, the value of $\Delta G^{LW}_{1W1}$ or $\Delta G^{LW}_{1W2}$ is only about $-0.5$ mJ/m$^2$ as the $\gamma^{LW}_1$ value of such hydrated entities is of the order of 27 mJ/m$^2$, versus 21.8 mJ/m$^2$ for $\gamma^{LW}_W$ of water [cf. Eqs. (2) and (3)]. In *aqueous media*, where essentially all Ag–Ab reactions take place, LW interactions are never the only long-range interaction forces, and usually represent less than 10% of the total interaction. The LW interaction energy thus is usually just a relatively small part of the total long- and medium-range interaction energy; it is additive to the other components. However, after expulsion of all interstitial water, LW interactions may suddenly account for virtually the entire short-range interfacial attraction energy.

### D. Minimum Distance Between Antigen and Antibody Surfaces and Decay with Distance of Lifshitz–van der Waals Forces

Owing to the marked increase in the LW attractions at short distances of interaction [see Eq. (1)], LW attractions are stronger, the better the steric fit between an epitope and its complementary paratope.* The minimum distance between the epitope and the paratope, as between other macromolecules or particles can be as small as about 2 Å (Pressman and Grossberg, 1968) and in ideal cases can approach 1.6 Å, which is of the order of magnitude of the minimum equilibrium distance $\ell_0$. Up to a distance $\ell \approx 100$ Å, $\Delta G^{LW}$ decays with $\ell$ in proportion to $(\ell_0/\ell)^2$ [see Eq. (2)]. However, at $\ell \geq 100$ Å, the rate of decay becomes steeper, due to retardation effects in the dispersion component of the LW forces. [For the decay with distance of LW forces in configurations, other than the plane-parallel configuration given in Eq. (1); see van Oss et al., 1988.] LW interaction energies between curved convex surfaces are proportional to the radius of curvature $R$.

## IV. POLAR, OR LEWIS ACID–BASE FORCES: HYDROGEN-BONDING INTERACTIONS

### A. Definition and Scope of Acid–Base, or Polar Interactions

Polar forces are designated as the forces operating in electron-acceptor–electron-donor, or Lewis acid–base interactions. These interactions are indicated by the superscript $AB$, for (Lewis) acid–base interactions. The superscript $AB$ used in this chapter does *not* stand for antibody; antibody is indicated by Ab, which is not used as a superscript. Hydrogen bonds (Brønsted acid–base

*The same reasoning holds for the other interaction classes: EL as well as AB forces also are both by far the strongest at the shortest possible distance, $\ell \rightarrow \ell_0$.

interactions) are a subclass of polar forces: all interactions that take place in a strongly hydrogen-bonding liquid, such as water, unavoidably comprise polar interactions.

## B. Polar, Acid–Base Free Energies

Polar energies also obey an equation based on the Dupré equation [see Eq. (2)], but the $\gamma_{ij}^{AB}$ term is expressed by an equation that is very different from the $\gamma_{12}^{LW}$ term:

$$\gamma^{AB} = 2(\sqrt{\gamma_i^+\gamma_i^-} + \sqrt{\gamma_j^+\gamma_j^-} - \sqrt{\gamma_i^+\gamma_j^-} - \sqrt{\gamma_j^+\gamma_i^-}) \tag{5}$$

where $\gamma_i^+$ and $\gamma_i^-$ are the electron-acceptor and the electron-donor parameters of the polar surface tension component $\gamma_i^{AB}$ where:

$$\gamma_i^{AW} = 2\sqrt{\gamma_i^+\gamma_i^-} \tag{6}$$

For homogeneous solids as well as for pure liquids, $\gamma_s^{LW}$, $\gamma_i^+$, and $\gamma_i^-$ can be determined [e.g., by contact angle measurements* (liquid–solid) or interfacial tension measurements (liquid–liquid)]. For whole biopolymers, all these parameters can be measured, both in the dry and in the hydrated state (van Oss et al., 1986a,b; van Oss, 1992). Unfortunately, however, a given *epitope* on a protein, or on a polysaccharide, is much too small for such measurements.

Based upon the Dupré equation for condensed media [cf. Eq. (2)], the polar (*AB*) free energy of interaction between different biopolymers (or cells), 1 and 2, immersed in an aqueous liquid, *W*, can be expressed as

$$\begin{aligned}\Delta G_{1W2}^{AB} = 2[&\sqrt{\gamma_W^+}(\sqrt{\gamma_1^-} + \sqrt{\gamma_2^-} - \sqrt{\gamma_W^-}) \\ &+ \sqrt{\gamma_W^-}(\sqrt{\gamma_1^+} + \sqrt{\gamma_2^+} - \sqrt{\gamma_W^+}) - \sqrt{\gamma_1^-\gamma_2^+} - \sqrt{\gamma_1^+\gamma_2^-}]\end{aligned} \tag{8}$$

## C. Hydrophobic or Attractive Acid–Base Interactions

The interaction between totally apolar (macro)molecules or particles, when immersed in water, is preponderantly (i.e., for ≈ 99%) polar and only for about 1% due to Lifshitz–van der Waals forces (van Oss and Good, 1991). These interactions are familiar to most under the name of *hydrophobic* interactions. There are tables listing the degree of hydrophobicity of various amino acids (Parker et al., 1986). These values, however, are only qualitatively linked to the (available) electron-acceptor ($\gamma^+$) and electron-donor ($\gamma^-$) parameters of given amino acids.

Averaging over the entire biopolymer, or cell surface, when $\Delta G_{1W2}^{AB}$ has a sufficient negative value to make the total value of $\Delta G_{1W2}^{TOT}$ ($= \Delta G_{1W2}^{LW} + \Delta G_{1W2}^{AB} + \Delta G_{1W2}^{EL}$) *negative*, a net attraction occurs, and when $\Delta G_{1W2}^{TOT}$ is *positive*, a net repulsion prevails. When a sufficient number of hydrophobic moieties are present in an epitope (EPI), *or* in a paratope (PARA)†; $\Delta G_{\text{EPI-water-PARA}}^{TOT}$ for such a pair will be negative, and a *specific* attraction can occur locally.

*This is done by contact angle (θ) determinations on dry (or on hydrated) layers of cells, proteins, or peptides, with three completely characterized liquids, *L* (two of which liquids must be polar), using the Young equation, which can be solved for the three unknown parameters $\gamma_S^{LW}$, $\gamma_S^+$, and $\gamma_S$ of the solid *S*:

$$(1 + \cos\theta)\gamma_L = 2(\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + \sqrt{\gamma_S^+\beta_L^-} + \sqrt{\gamma_S^-\gamma_L^+}) \tag{7}$$

This equation should be used three times, with the different values for θ obtained with the three different liquids, *L* (van Oss et al., 1988).

†It should be stressed that, for a hydrophobic attraction to occur in aqueous media, it suffices for just one of the sites to be hydrophobic; the other site may be fairly hydrophilic.

## D. Hydrophilic, or Repulsive Acid–Base Interactions

On the other hand, hydrophilic macromolecules or cells, when immersed in water, manifest a net polar (AB) repulsion, averaged over their total contactable surface. In these cases, $\Delta G_{1W2}$ ($= \Delta G^{LW}_{1W2} + \Delta G^{AB}_{1W2}$) is *positive* (even though $\Delta G^{LW}_{1W2}$ is negative). This repulsion, which is predominantly due to AB forces, is the principal driving force for the solubility of proteins and for the stability of cells in aqueous media (van Oss, 1993).

## E. Decay of Polar Acid–Base Forces as a Function of Distance

Polar forces decrease as a function of distance at an exponential rate. The rate of decay with distance of $\Delta G^{AB}$ is expressed (in the configuration of parallel bodies) as:

$$\Delta G^{AB}_{l} = \Delta G_{l_0} \exp[(\ell_0 - \ell)/\lambda] \tag{9}$$

where $\ell_0$ is the minimum equilibrium distance (which may be taken to be of the same order of magnitude as for LW interactions; that is, $\ell_0 \approx 1.6$ Å), and where $\lambda$ is the correlation length (or decay length) typical for the solvent molecules which, for liquid water, has an empirical value of the order of 0.6–1.0 nm (van Oss, 1993). Contrary to electrostatic interactions (see below), the decay of AB forces with distance is not influenced by the ionic strength of the liquid medium. For the decay of AB energies in configurations other than plane-parallel (see van Oss et al., 1988), the interaction energies between curved convex surfaces are proportional to the radius of curvature, $R$.

# V. ELECTROSTATIC FORCES

## A. Nature of Electrostatic Forces

Electrostatic (EL), or Coulombic, interactions between Ag and Ab are due to the presence of one or more ionized sites on the epitope and oppositely charged sites on the paratope. These typically are the $COO^-$ and the $NH_2^+$ or $NH_3^+$ groups on the amino acids of the Ag and Ab molecules (where the Ag is a protein or a peptide), or similarly charged moieties on carbohydrate or other nonproteinaceous Ags.

## B. Electrostatic Free Energies

In most in vivo and also in in vitro situations, biopolymers and cells, being generally negatively charged, usually are subject to at least a small amount of mutual EL repulsion. The main measurable property of the overall surface charge of a biopolymer or cell, is its surface (or $\zeta$) potential. This is most easily measured by electrophoresis and from the $\zeta$-potential, thus obtained, the fundamental potential ($\psi_0$-potential) at the exact particle (or molecule)–liquid interface can be obtained. From the $\psi_0$-potential, $\Delta G^{EL}$ as a function of distance, $\ell$, can be obtained as follows:

$$\Delta G^{EL}_{(l)} = 1/\kappa \cdot 64\, nkT\gamma_0^2 \exp(-\kappa\ell) \tag{10}$$

where $1/\kappa$ is the Debye length, or the electrostatic decay length:

$$1/\kappa = \sqrt{\epsilon kT/(4\pi e^2 \Sigma \nu_i^2 n_i^2)} \tag{11}$$

and

$$\gamma_0 = \frac{\exp(ve\psi_0/2kT) - 1}{\exp(ve\psi_0/2kT) + 1} \tag{12}$$

[$\epsilon$ = dielectric constant of the liquid (for water, $\epsilon$ = 80); $k$ = Boltzmann's constant ($k$ = $1.38 \times 10^{-23}$J per °K); $T$ = the absolute temperature in °K; $e$ = charge of the electron ($e$ = $4.8 \times 10^{-10}$ e.s.u.); $v_i$ = the valency of each ionic species in solution in the liquid; $n_i$ = the number of ions of each species per cubic centimeter ($cm^3$) of bulk liquid; $\nu$ = the valency of the counterions dissolved in the liquid] (see, e. g., Overbeek, 1952; Overbeek and Bijsterbosch, 1979; Hunter, 1981). Equation (10) pertains to the interaction between two plane-parallel bodies; for other configurations see, for example, van Oss et al. (1988). It should be noted that for the EL interaction between spheres with radius, $R$, $\Delta G^{EL}$ is proportional to $R$, and inversely proportional to $\psi_0^2$.

## C. Decay of Electrostatic Forces as a Function of Distance

In general, the rate of decay of EL interactions with distance may be expressed as:

$$\Delta G_l^{EL} = \Delta G_{l_0} \exp(-\kappa \ell) \tag{13}$$

It is clear from Eqs. (10) and (11) that the value $\Delta G^{EL}$ strongly depends on the ionic strength [see $n_i$ of Eq. (11)] of the liquid medium: for a given value of $\psi_0$, $\Delta G^{EL}$ has the highest absolute value at the lowest salt content. The values for $1/\kappa$ are for 0.1 *M* NaCl: 10 Å; for 0.01 *M* NaCl: 100 Å; and for $10^{-5}$ *M* NaCl: 1000 Å. At physiological salt concentration (i.e., 0.15 *M* NaCl), $1/\kappa \approx 8$ Å. Thus, in more dilute salt solutions, EL interactions are measurable at much greater distances than at high ionic strengths [see Eq. (10)].

From Eqs. (9) and (10) it can be seen that both AB and EL free energies decay exponentially as a function of distance, $\ell$, and of a constant ($\lambda$, resp. $1/\kappa$). In the case of AB forces, that constant ($\lambda$) is relatively invariable and mainly linked to the molecular properties of the liquid (e.g., water). In the case of EL forces, that "constant" ($1/\kappa$) is variable; it changes with the concentration (and kind) of salts dissolved in the (aqueous) liquid. Thus $|\Delta G^{EL}|$ not only is lowest at high salt concentration, it also decays much more steeply at high ionic strengths.

In the interaction between erythrocytes [which have a moderately high $\psi_0$ potential of about −26 mV], the EL repulsion (acting at a distance) between cells (albeit weaker than the AB repulsion), contributes to the total intercellular repulsion (and thus to the stability of red cell suspensions) in a nonnegligible manner. The ionic strength of the aqueous medium not only influences erythrocyte stability; it also governs hemagglutination in other ways due to the variation of the EL interaction energy with distance, as a function of ionic strength (see Chap. 28).

## D. Calcium Bridging

Purely (or mainly) electrostatic Ag–Ab interactions can occur not only through negatively charged sites on one determinant attracting positive sites on the other, but also via the binding of negatively charged epitopes to equally negatively charged paratopes by means of linkage through, for example, $Ca^{2+}$ ions, by analogy with cell–cell interaction and cell–adhesion phenomena. One example of $Ca^{2+}$-bridging, involving synthetic polypeptides (comprising negatively polyglutamic acid moieties), has been described by Liberti (1975). Ag–Ab complexes of that type can be dissociated with the complexing agent ethylenediaminetetraacetic acid (EDTA). Other $Ca^{2+}$-dependent reactions have been observed by Kumar (unpublished observation, 1989) in DNA–anti-DNA interactions, and by Prickett et al. (1989), in Abs to a marker peptide used in the affinity purification of recombinant proteins.

More recently, it has become apparent that the interaction between negatively charged entities, upon the admixture of plurivalent cations such as $Ca^{2+}$ is only partly of an electrostatic nature. Calcium not only acts in "bridging" (albeit to a fairly slight extent), or in reducing the

$\zeta$-potential of the negatively charged entities, but it also acts as an electron-acceptor which can strongly attenuate the electron-donicity of such (negatively charged) hydrophilic entities, thereby making them *hydrophobic*. The principal effect of the admixture of plurivalent cations is their power to convert negatively charged hydrophilic surfaces into less negatively charged *hydrophobic* surfaces (van Oss et al., 1993; van Oss, 1994b).

Plurivalent cations also play a role in other specific interactions [see, e.g., the role of $Ca^{2+}$ and of $Mg^{2+}$ in different parts of the complement cascade (Morgan, 1990; see also Chap. 34], and the requirement for $Ca^{2+}$ in enzymatic reactions (e.g., phosphorylase kinase), and the blood clotting cascade (prothrombin); see, e.g., Stryer, 1981.

## VI. SIZE OF BINDING SITES

### A. Smallness of Epitopes and Paratopes

When measured by inhibition experiments, with small peptides, in proteins and peptides, the maximum size of the epitope was found to be close to that of penta- or hexapeptides, yielding a specific surface area varying between 2.5 and 5 $nm^2$ (Kabat, 1976; Atassi, 1984). However, specific epitope (or hapten) surface areas as small as 0.4–2.5 $nm^2$ can also occur, whilst areas up to about 10 $nm^2$ also have been described (see, e.g., Amit et al., 1986; Davies et al., 1990) (Table 1). Novotny et al. (1989) believed that only a relatively small number of amino acids within the epitope and paratope participate in Ag–Ab binding. In polysaccharides, the maximum size of epitopes is close to that of penta- or hexasaccharides, corresponding to the same maximum specific surface area as that for peptides. Here also, much smaller specific epitope surface areas can occur (Atassi, 1984; see also Table 1). When large surface areas (e.g., areas of 20 $nm^2$ or more) are found for an epitope, the formation of secondary bonds should be considered likely (see Sec. XI.D). It should also be emphasized that the apparent size of an epitope, and even its definition, is closely linked to the approach used in its measurement (Van Regenmortel, 1989; Greenspan, 1992).



**Table 1** Comparisons Between Aspecific (Cell–Cell, Cell–Biopolymer, or Biopolymer–Biopolymer) and Specific (Ag–Ab) Interactions

| | Aspecific | Specific |
|---|---|---|
| Nature of contact sites | Usually rather homogeneous | Often heterogeneous |
| Surface area of contact | Large (10–100 $nm^2$) | Small (0.4–10 $nm^2$) |
| Total size of molecule or particle | May be quite large (up to 10 $\mu$m in diameter) | Usually small (1–10 nm Stokes radius, or with processes of a very small radius of curvature) |
| Influence of Brownian movement | Small | Moderate to small |
| Binding energy | May be repulsive or attractive, but is usually repulsive (+25 $mJ/m^2$[a], for erythrocytes) | Attractive (−7 to −25 kT[b] per particle, or −10 to −50 $mJ/m^2$) |

[a]Strong repulsive forces of this magnitude can only be overcome by moieties with a very small radius of curvature, hence the smallness or thinness of most molecules comprising a ligand or receptor.

[b]$k$ is Boltzmann's constant ($k = 1.38 \times 10^{-23}$ J per °Kelvin) and $T$ is the absolute temperature in Kelvin. Thus, at 20°C (= 293°K), 1 kT = $4.04 \times 10^{-21}$ J.

*Source*: Adapted from van Oss (1990b).

It is obvious that the surface areas of epitopes are closely comparable with those of the corresponding paratopes.

## B. Advantage of Multiple Binding Sites

The crucial advantage of the smallness of the specifically reacting moieties or macromolecules is that they can overcome the normally prevailing repulsion field any time that aspecific repulsion is smaller than the specific attraction. Concomitantly, the energy of binding of small sites is also small, but this can be overcome in the following manner: Significant attraction between small receptors (e.g., Fc receptors on cells) and ligands (e.g., Fc tails of antibody molecules) will occur when, after complex formation between several Ab molecules and an Ag, *several* Fc tails can attach to Fc receptors on one cell (van Oss et al., 1985a). Thus if the binding constant of one Fc tail to one Fc receptor, $K_{ass} = 10^4$ (mol fraction)$^{-1}$, then, for the interaction of an Ag–Ab complex, which can attach to a cell with three Fc tails simultaneously, the binding constant $K_{ass}$ for the complex jumps to $10^{12}$L/*M*, according to:

$$\Delta G_{1W2}^{TOT} = -kT \ln K_{ass} \tag{14}$$

where $kT = 4.04 \times 10^{-21}$J (at 20°C) (see also Karush, 1976 and Chap. 30). Table 2 shows the correlation between binding energies in kcal/mol, kT units, and $K_{ass}$ in (mol fraction)$^{-1}$.* This amplification mechanism explains why Ag–Ab complexes with one or two Ab molecules remain largely unnoticed by the phagocytic system, and only Ag–Ab complexes with three or more Ab molecules readily become phagocytized (van Oss et al., 1984). For the same reason, biological recognition molecules for Fc have an advantage in being multivalent: the first serum complement factor, Clq, is hexavalent (i.e., it has six separate sites, each one of which can bind one Fc moiety of an immunoglobulin molecule). This is essential, because Clq molecules circulate freely in the bloodstream, surrounded by an excess of free immunoglobulin molecules, which it must not, in the normal course of events, bind irreversibly. Only a much more irreversible binding of complexes comprising immunoglobulins, to two or more of the binding sites of Clq activates the complement cascade (Cooper, 1985; Morgan, 1990; see also Chapter 34).

## C. Difficulty of Predicting Binding Energies from Physical Measurements on Binding Sites

One drawback of the smallness of specific sites is that, contrary to aspecific interactions, the contributions of the separate constituent chains cannot be measured directly by contact angle or electrokinetic determinations. This is due to the smallness of the actual specifically interacting determinants, which usually have an interacting surface area of the order of only 0.4–10 nm$^2$, representing a bare fraction of 1% of the total surface area of a specific biopolymer (e.g., the two paratopes of an antibody of the IgG class), or of the biopolymer which it can specifically recognize (e.g., one epitope of a serum albumin molecule). Also, even if in some cases, one can readily isolate the specific epitope (or more rarely, paratope) molecules, due to the pronounced variability of the detailed molecular composition of the epitope or paratope, contact angle or electrokinetic measurements on such isolated moieties will usually still yield little information on the type and strength of the binding modes of each of the individual constituent monomers. In addition, decomposing the epitopes or paratopes into their constituent monomers will yield neither the relative contribution of each type of bond, nor the quantitative bond strength of each monomer

*For important developments related to Table 2 see note added in proof, page 610.

**Table 2** Bond Energy, $\Delta G$, at 20°C, at Contact, Expressed in kcal/mol, Units of kT (1 kT = $4.05 \times 10^{-21}$ J/molecule at 20°C) and in Terms of the Corresponding Binding Constant [see Eq. (14)]

| $\Delta G$ | | | $\Delta G$ | | |
|---|---|---|---|---|---|
| kcal/mol | kT | $K_{ass}$ [(mol fraction)$^{-1}$][e] | kcal/mol | kT | $K_{ass}$ [(mol fraction)$^{-1}$][e] |
| −0.58[a,b] | −1 | 2.7 | −8.15[d] | −14 | $1.2 \times 10^6$ |
| −1.17[a,b] | −2 | 7.4 | −8.73[d] | −15 | $3.3 \times 10^6$ |
| −1.75[a,b] | −3 | 20.1 | −9.31[d] | −16 | $8.9 \times 10^6$ |
| −2.33[a,b] | −4 | 54.6 | −9.90[d] | −17 | $2.4 \times 10^7$ |
| −2.91[a,b] | −5 | $1.5 \times 10^2$ | −10.48[d] | −18 | $6.6 \times 10^7$ |
| −3.49[a,c] | −6 | $4.0 \times 10^2$ | −11.06 | −19 | $1.8 \times 10^8$ |
| −4.08[a,c] | −7 | $1.1 \times 10^3$ | −11.64 | −20 | $4.9 \times 10^8$ |
| −4.66[c] | −8 | $3.0 \times 10^3$ | −12.22 | −21 | $1.3 \times 10^9$ |
| −5.24[c] | −9 | $8.1 \times 10^3$ | −12.81 | −22 | $3.6 \times 10^9$ |
| −5.82 | −10 | $2.2 \times 10^4$ | −13.39 | −23 | $9.7 \times 10^9$ |
| −6.40 | −11 | $6.0 \times 10^4$ | −13.97 | −24 | $2.7 \times 10^{10}$ |
| −6.98[d] | −12 | $1.6 \times 10^5$ | −14.55 | −25 | $7.2 \times 10^{10}$ |
| −7.57[d] | −13 | $4.4 \times 10^5$ | | | |

[a]Energy range of one direct H bond (Eisenberg and Kauzmann, 1969).
[b]Energy range of the hydrophobic attraction ($\Delta G^{AB}_{1W2}$, *before* expulsion of water, but nevertheless expressed at "contact", i.e., at $\ell \rightarrow \ell_o$), per $Sc = 0.4$ nm$^2$. This is also the energy range of the electrostatic attraction ($\Delta G^{EL}_{1W2}$, also *before* expulsion of water but, again, expressed at "contact").
[c]Energy range of the interfacial attraction at closest contact ($\Delta G^{LW}_{12}$, *after* expulsion of water).
[d]Energy range of one direct electrostatic bond ($\Delta G^{EL}_{12}$), after expulsion of water (e.g., between $COO^-$ and $NH_3^+$) (Gabler, 1978). However, before extrusion of water, $\Delta G^{EL}_{1W2}$ is only 1–3 kT (see b above).
[e]1 mol/L = 0.018 mol fraction (because there are 55.56 mol $H_2O$ in 1 L), and 1 L/mol = 55.56 (mol fraction)$^{-1}$. Thus, to obtain $K_{ass}$ in L/mol, each figure in this column must be divided by a factor of 55.56, to correspond to the bond energy ($\Delta G$) in the same row.
a, c, and d indicate direct and final interaction energies; b shows the AB and EL energies important at medium- to short-range, from 2.0 to about 0.2 nm (see Table 4).



(e.g., of each amino acid in a peptide) vis-à-vis the opposing monomer. Even if one could synthesize monotonous polypeptides for each amino acid of a given epitope, and measure their $\gamma^{LW}$, $\gamma^{\oplus}$, $\gamma^{\ominus}$ values and their $\zeta$-potential to obtain their energetic contributions to the total specific bond, the problem still would not be solved. This is because one cannot add up all these contributions to obtain the total binding energy of the epitope, without situating each of them precisely into the three-dimensional configurations that are known for only a handful of antigens. In addition, all these properties also have to be ascertained for the corresponding paratope, and the two (epitope and paratope) then must be precisely fitted together in a three-dimensional array. Unfortunately, the three-dimensional (and even the linear) configurations of paratopes are known in only a few cases. Work is proceeding slowly to unravel the three-dimensional configuration of a few intermolecular epitope–paratope interactions (Amit et al., 1986; Davies et al., 1990), but little, if anything, has yet been done to measure the value of each of its different contributing physical interaction forces. For the time being, indirect approaches remain the sole means by which one can estimate the contribution to specific interactions of the different types of intermolecular forces (van Oss, 1990b). A number of examples of such indirect approaches are given in the Appendices to this chapter.

## VII. SPECIFIC INTERACTIONS AT A DISTANCE

### A. The Obligatory Long-Range Attraction Between Epitope and Paratope (and Between Other Ligands and Receptors)

The fact that, in the normal course of events, all cells and biopolymers immersed or dissolved in aqueous media under physiological conditions, repel one another and thus usually cannot approach each other more closely than to about 3–8 nm, makes it a prerequisite for specifically interacting ligand and receptor sites to be able to attract each other from a distance (Fig. 1). Two factors can contribute to make this possible: (1) the specifically attracting sites are usually small, and at least one of them (usually the epitope) is prone to lie on a prominent edge or peak (cylinder or sphere) with a small radius of curvature $R$, which enables it to pierce the general repulsion field; and (2) the two sites either have a significant electric charge of opposite signs, *or* at least one of them is hydrophobic (in which case the other one may be hydrophilic, but not excessively so). Once the repulsion field is pierced, the epitope–paratope bond strengthens, either through the sheer effect of the smaller distance between the two, or because of a qualitative change in the nature of the bond; for example, through expulsion of water of hydration, or (probably relatively rarely) through formation of direct hydrogen bonds. Also, once epitope and paratope have approached each other closely, in many cases, secondary bonds can ensue between biopolymer moieties which are in close vicinity to epitope and paratope. In Table 2 the energy ranges are shown of the various interaction types. Table 3 shows, in the left-hand column, the quantitative contributions to the Ag–Ab bond.

### B. Long- and Medium-Range Specific Lifshitz–van der Waals Interactions

As shown in Table 3, the long- and medium-range LW interaction between specific sites of 0.4 nm$^2$ contactable surface ($S_c$) area* is quite weak, being of the order of 0.5–0.5 kT, calculated at close range, and even 20 times smaller at a distance of only 1 nm. Thus, these LW forces usually are so small that they cannot be shown in Table 2.

Nevertheless, in some instances, even weakly attractive LW forces can, in the absence of EL forces, achieve Ag–Ab binding (see the case of dextran described in Appendix I). The attraction here is for 91% due to LW and for 9% to AB forces. Beyond about 2 nm distance, the attraction decreases to less than 1 kT, but at "contact" it is of the order of $-10.7$ kT, due in part to the fairly large value for $S_c$, of 6 nm$^2$.

### C. Long- and Medium-Range Specific Acid–Base Interactions

Whilst repulsive AB forces or "hydration pressure," represent the principal mode of repulsion between biopolymers or cells in aqueous media, *attractive* AB forces, or "hydrophobic interactions" are one of the two main mechanisms of specific attraction between epitopes and paratopes, as well as between other ligands and receptors (the other mechanism being the EL interaction, see below). Most frequently, in epitope–paratope interactions of the attractive, hydrophobic (AB) variety, the hydrophobic moiety in the interacting partnership tends to be located on the paratope, and is then usually situated in a concavity ("cleft") of the paratope.

*A value for $S_c$ of 0.4 nm$^2$ (= 40 Å$^2$) is taken as the minimum standard unit contactable surface area, to facilitate the quantitative comparison between different types of forces. Smaller $S_c$ values are rare (see, however, Appendix III), but $S_c$ values up to 10 nm$^2$ (= 1000 Å$^2$) can occur (see Table 1).

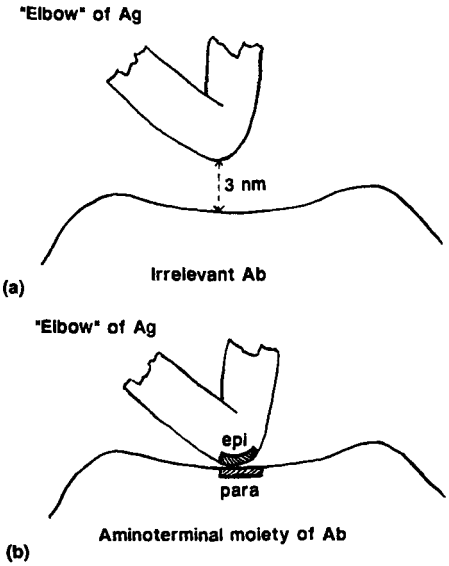


**Figure 1** Schematic presentation stressing the difference between the interaction of an antigen with an irrelevant antibody molecule and the interaction of an antigen with its corresponding antibody. (a) When no specific attraction exists between an Ag and an irrelevant immunoglobulin, an "elbow" of the Ag molecule can come closest to the immunoglobulin surface, but not closer than about 3 nm. (b) When there is a specific medium-range attraction between an epitope on the "elbow" of an Ag and the paratope on the Ab, the averaged macroscopic long- and medium-range repulsion between Ag and Ab can be surmounted and a close-range Ag–Ab bond ensues.



The immunodominant epitopes of proteins tend to lie on prominent, predominantly hydrophilic peptide loops [see, e.g., Atassi (1984) and Colman (1988), who stresses the requisite for surface accessibility, and Hopp and Woods (1983), who equate hydrophilicity with immunodominance of epitopes]. It should be recalled that overall hydrophilicity is a prerequisite for the prominent placement of peptide moieties at a protein's aqueous interface. However, hydrophobic amino acids can and do occur in immunodominant epitopic sites (Getzoff et al., 1988), although such epitopes tend to comprise only a few hydrophobic amino acids (cf. Atassi, 1984; Geysen et al., 1988; Novotny et al., 1989). Amino acids of low polarity occur even more commonly in paratopic sites (Padlan, 1992; Kelley and O'Connell, 1993). In paratopes such hydrophobic moieties tend to be situated in concavities (or "clefts"), which obviates undue aspecific hydrophobic interactions with irrelevant biopolymers (van Oss, 1990b).

An example of a mainly hydrophobic epitope–paratope interaction is the reaction between the hapten, nitrobenzene, and its paratope (see Appendix II). Here the (close-range) energy of attraction of about $-8$ kT is almost 90% of polar, AB origin. With relatively feebly hydrophobic interactions, the attraction drops below 1 kT beyond a distance of 2.5 nm, but for stronger hydrophobic interaction, such as between the nitrobenzene epitope and its paratope, the attraction still is of the order of 1 kT, at a distance of 20 nm, as a consequence of a longer decay length for water in very hydrophobic systems (see Appendix II). The $S_c$ area for this system is about

**Table 3** Long-Range vs Short-Range Action of Specific Ag–Ab Bonds

| Long- and medium-range bonds | Exclusively short-range bonds |
|---|---|
| A. Lifshitz-van der Waals (LW) interactions: $\Delta G^{LW}_{1W2}$. Relatively weak interactions. (About 0.5–0.05 kT[a] per 0.4 nm$^2$); see also E, in the right-hand column. | D. Direct H bonds. Not very strong, occur only rarely. (About $-1$ to $-7$ kT per bond). |
| B. Polar (AB) interactions: $\Delta G^{AB}_{1W2}$. Relatively strong interactions (about $-1$ to $-5$ kT per 0.4 nm$^2$), but AB component becomes very weak on switching to $\Delta G_{12}$ after extrusion of water. However, $\Delta G_{12}$ then represents mainly $\Delta G^{LW}_{12}$, which is a rather strong rather strong interaction. | E. Short-range interfacial bonds: $\Delta G_{12}$. Occurring after extrusion of interstitial water molecules: mainly $\Delta G^{LW}_{12}$. Rather strong interaction (about $-6$ to $-8$ kT per 0.4 nm$^2$). |
| C. Electrostatic (EL) interactions: $\Delta G^{EL}_{1W2}$. Relatively tively strong interactions (About $-1$ to $-3$ kT per site) on an average, and at a distance. Between strongly charged (oppositely charged) sites this can be as high as $-5$ to $-12$ kT, and upon extrusion of water becomes $\Delta G^{EL}_{12}$, which is stronger still. | F. Short-range EL bonds: $\Delta G^{EL}_{12}$. Strong interactions (about $-12$ to $-18$ kT per bond). |

[a]1 kT = 0.58 kcal/mol (see Table 2). The energy ranges given are at "contact." At distances $\ell > \ell_0$, the energies become correspondingly smaller [cf. Eqs. (2), (9), and (10)].



0.7 nm$^2$. Approximately the same types and strengths of binding energies that apply to nitrophenol, also apply to dinitrophenol (DNP) epitopes (see Appendix II).

There is a widespread belief that hydrophobic interactions are mainly entropic (i.e., mainly represented by $T\Delta S$) according to:

$$\Delta G = \Delta H - T\Delta S \tag{15}$$

where $\Delta G$ is the total free-energy difference of a system, and $\Delta H$ and $T\Delta S$ represent its enthalpic and entropic components, respectively, $T$ is the absolute temperature in K (see also Chap. 30). For alkanes immersed in water, this is indeed largely true (Tanford, 1980). However, it can be demonstrated that, for other apolar compounds or for compounds of low polarity immersed in water, this is by no means the rule: their interaction can be mainly enthalpic or mainly entropic, or partly one and partly the other, depending on each individual case (van Oss and Good, 1991).

## D. Long- and Medium-Range Specific Electrostatic Interactions

Whilst average surface potentials of 25 mV or more are quite rare for most cells or biopolymers on a macroscopic scale, on a smaller scale, specific sites can locally have potentials up to several hundred millivolts (mV), within a surface not larger than 1 nm$^2$. Thus specific sites may attract each other with EL energies of $-1$ to $-6$ kT extrapolated to "contact," but not at real contact (see below) so that, at best, at distances greater than 2 nm, $\Delta G^{EL}$ usually becomes less than 1 kT. Electrostatic interactions causing the specific attraction between epitope and paratope are quite common. EL interactions play a role with many protein Ags [e.g., bovine serum albumin (BSA); see Appendix IV; van Oss et al., 1982] and with a great many haptens, such as benzoates, arsonates, amines, phosphonates, anilates, hippurates, phthalates, propionates, and others (Pressman and Grossberg, 1968). As shown for BSA epitopes (see Appendix IV), it may take three more or less ionized neighboring basic amino acids to represent one fully charged $NH_3^+$ ion.

It should be stressed that one often finds one of the immunodominant epitopes of a protein

in the close vicinity of the carboxyterminal; this occurs, for instance, in myoglobin and serum albumin (see, e.g., Atassi, 1984). Paratopes, on the other hand, are always close to the $NH_2$-terminals of immunoglobulins. This suggests that a medium- to long-range EL attraction between the carboxyterminal of an Ag and the corresponding Ab may be a fairly common occurrence. Even in small peptides this observation may be important: it was recently shown that it was only possible to elicit antibodies to the tetrapeptide, Tuftsin, when the peptide was linked to the carrier protein with its carboxyterminal pointing outward (Naim and van Oss, 1991).

## E. Implications for the Shape and Size of the Epitope

Cells (e.g., circulating blood cells) suspended in physiological fluids will not approach each other more closely than to within 5.0 nm. This is the distance between the outer edges of the glycocalices of two red blood cells which still repel each other with an energy of +1 kT. At an even slightly closer approach, the repulsive energy increases exceedingly steeply: at 3 nm the repulsion between two red cells would be +100 kT (see Fig. 5 in Chapter 28). The same holds true for most proteins (e.g., blood serum proteins); they can approach each other somewhat more closely because of the smaller radius of curvature resulting from their much smaller size (compared with cells). However, proteins usually cannot approach each other more closely than to within 3.0–3.5 nm. On the other hand, specific attraction energies between ligands and receptors (e.g., epitopes and paratopes) decay to less than 1 kT at distances greater than about 2.0 nm (Table 4). Thus, at first sight, it would not seem possible for specific attraction energies (which usual quickly become negligible at distances greater than 2.0 nm) to overcome the sizeable macroscopic repulsion energies between cells or proteins (which grow increasingly enormous at distances less than 3.0 nm). However, cell and biopolymer surfaces do not actually display the total molecular surface smoothness upon which the aforementioned energy versus distance analyses are based. In reality, on a molecular level, the surfaces of cells and biopolymers more often are spiculated, nodulated, or corrugated. Now, the immunodominant epitopes of whole intact cellular and biopolymeric antigens tend to be situated precisely on those moieties with the smallest radii ($R$) of curvature, of these spicules, nodules, or ridges. Thus, whilst the *average* surfaces of antigenic particles or molecules cannot approach the surfaces of antibody molecules closely enough to overcome the general repulsion field, the epitopes situated on protruding processes with a small $R$ *can* achieve contact, *provided a specifically complementary attracting paratope is encountered*. Even then, the relatively aspecific AB or EL forces at work in the medium- to short-attraction range of 2 nm to contact at somewhat under 0.2 nm, need not give rise to a stronger preliminary bond than a few kT units, whilst an epitope–paratope bond which is



**Table 4** Decay with Distance of Long- and Medium-Range-Specific Interactions

| | Energy $\Delta G_{1W2}$ in kT,[a] at distance, $\ell$, in nm | | | |
|---|---|---|---|---|
| Bonds | 1 nm | 2 nm | 3 nm | 4 nm |
| LW: max.: ≈ 0.5 kT per 0.4 $nm^2$ (expressed at contact) | 0.08 | 0.04 | 0.03 | 0.02 |
| AB (hydrophobic): max.: ≈ 5 kT per 0.4 $nm^2$ (expressed at contact) | 2.2[b] | 0.8[b] | 0.3[b] | 0.1[b] |
| EL: max.: ≈ 5 kT per site (expressed at contact) | 1.7 | 0.5 | 0.1 | 0.04 |

[a]For larger contactable surface areas than $S_c$ = 0.4 $nm^2$ (e.g., $x$ times 0.4 $nm^2$), the $\Delta G_{1W2}$ values in this table must be multiplied by the factor $x$.

[b]For more strongly hydrophobic surfaces, the decay with distance of attractive AB forces is much more gradual, so that all these values are much higher (see, e.g., the case of the $NO_2$ epitope, described in Appendix II).

likely to persist should at least have an energy of 10 kT, and preferably from 12 to 18 kT or more. Such bond energies are generally only attainable after expulsion of interstitial water molecules of hydration (i.e., under conditions of closest "fit"; see the following section).

## VIII. CLOSE-RANGE SPECIFIC INTERACTIONS

### A. The Role of Hydration

All surfaces in contact with water (with the exception of air) become hydrated with *energies of hydration*, $\Delta G_{iw}$, varying from $-40$ to $-113$ mJ/m$^2$ for hydrophobic (i.e., water-insoluble compounds) to $-114$ to about $-140$ mJ/m$^2$ for hydrophilic, water-soluble materials (van Oss, 1992; 1994a). *In terms of free energy per unit surface* the usual hydration $|\Delta G_{iw}|$ values for epitopes and paratopes are higher than the epitope–paratope-binding energy, $|\Delta G_{1W2}|$, so that one might at first sight believe that both epitope and paratope have to keep at least one molecular layer of water of hydration even after binding. This is not so, but if it were, the epitope–paratope-binding energy ($\Delta G_{1W2}$), at $\ell \geq 0.7$ nm, would be very small (e.g., of the order of only $-1$ kT), which would not lead to an effective bond.

It should be noted first that for even the most hydrophilic materials known, the free energy of hydration ($\Delta G_{iw}$) is smaller than the free energy of cohesion between the water molecules themselves: the strongest energy of hydration known (i.e., that of polyethylene oxide, with $\Delta G_{iw} = -140$ mJ/m$^2$) still is smaller than the energy of cohesion of water ($\Delta G_{ww}$), which is equal to $-142.8$ mJ/m$^2$. But given a contactable surface area, $S_c$, for single water molecules of about 0.1 nm$^2$, $\Delta G_{ww} = -142.8$ mJ/m$^2$ *still equals only* $-3.5$ *kT*. All energies of hydration thus are smaller than 3.5 kT. At the same time, the free energies of binding between epitopes and paratopes generally are from at least $-7.5$ to $-10$, to as much as $-12$ to $-25$ kT (corresponding to $K_{ass}$ values of $1.8 \times 10^4$ to $7.2 \times 10^{10}$ L/*M* [see Table 2 and Eq. (14)]. Thus upon epitope–paratope binding, water of hydration can be largely extruded, if the "fit" is good enough. At a less than perfect fit however, water cannot be expelled from gaps where otherwise a vacuum would form. Because the water of hydration, especially on hydrophilic molecules, tends to be strongly oriented, loss of such oriented water of hydration into the randomly oriented bulk liquid gives rise to a marked increase in the entropy of the system. This is the main cause of the typical increase in entropy observed in many Ag–Ab reactions (Absolom and van Oss, 1986; van Oss, 1992). However, all water molecules of hydration are not necessarily always extruded. Some water may remain if there is a less than perfect "fit", and in other cases tightly bound water molecules have been thought to participate in the achievement of an optimal "fit" (Pressman and Goldberg, 1968, pp. 132–137; see also Fischman et al., 1991). In general, however, the presence of residual interstitial water will locally reduce the binding energy between epitope and paratope rather severely, because they then interact at a greater than minimum distance.

With increasing temperature, there is little change in the total Ag–Ab binding energy, but there is an increase in $T\Delta S$, which compensates almost completely for a concomitant decrease in $|\Delta H|$ [see Eq. (15)] (van Oss et al., 1982; Mukkur, 1984). This enthalpy–entropy compensation may be qualitatively explained by the following mechanism: At higher temperatures, more dehydration of the various (peptide) chains takes place, which causes expulsion of previously oriented water molecules of hydration into the bulk liquid, which entails an increase in $T\Delta S$ and a decrease in the (negative) value of $\Delta H$. This also allows Ag and Ab chains to approach each other more closely, giving rise to a moderate increase in (mainly secondary) "hydrophobic" attraction or to a further (secondary) change from the $\Delta G_{1W2}$ to the $\Delta G_{12}$ mode (see below). This also compensates, or even slightly overcompensates, for the decrease in the Ag–Ab-binding energy which normally should accompany an increase in temperature, inter alia as a consequence of an increase in the $T$ of kT.

Positive $T\Delta S$ values (which frequently occur in Ag–Ab interactions) are plausibly linked to the randomization of previously oriented water molecules of hydration, lost into the bulk liquid after the encounter of Ag with Ab. However, the occurrence of *negative* $T\Delta S$ values is also not uncommon (van Oss and Absolom, 1986; van Oss, 1992b); they possibly arise in part from a more orderly rearrangement of $V_H$ and $V_L$ domains in the paratope (Tello et al., 1993). In some cases negative $T\Delta S$ values have also been known to result from hydrophobic interactions (van Oss and Good, 1991).

## B. The $\Delta G_{1W2} \rightarrow \Delta G_{12}$ Transition: Expulsion of Water

Dehydration of the epitope–paratope interface, as exemplified by the $\Delta G_{1W2} \rightarrow \Delta G_{12}$ transition, and as manifested by the increase in entropy typically observed in Ag–Ab and other specific interactions, is one of the most important aspects of the secondary (or "ripening") part of the specific bond. As a consequence of dehydration, and of the optimum "fit" between epitope and paratope, the necessary binding energy, of the order of 10 kT or more, can be reached, even with a small surface area of the specific epitope–paratope interface.

Thus in many, if not in most cases, the secondary binding due to dehydration (i.e., the $\Delta G_{1W2} \rightarrow \Delta G_{12}$ transition) is crucial for the ultimate strength of the specific bond. As an example, we may again revert to the dextran–antidextran system, already discussed above in terms of $\Delta G_{1W2}$, where at least four of the glucose units, in the α-(1→6) linkage are of primary importance in the Ag–Ab bond, and two more are needed to achieve the total-binding strength (Kabat, 1976, p. 126); see Appendix I. However, it should also be realized that, with dextran as well as with other polysaccharides (branched or otherwise), the terminal nonreducing sugar residue often is an even more important immunodominant moiety (Kabat, 1976; p. 126). The nonreducing half of glucose is probably the "primary point of attachment" to the epitope (Kabat, 1976, p. 125; see Appendix III). For a contactable surface area, $S_c$, of only 0.2 nm$^2$ for the nonreducing moiety of the glucose molecule (for which the entire glucose molecule has an $S_c$ value of 0.4 nm$^2$; van Oss, 1984a), this corresponds to $\Delta G_{12} = -4.5$ kT, or $-2.6$ *kcal/mol*, which correlates well with the contribution of the immunodominant glucose moiety of $-2.9$ kcal/mol given by Kabat (1976, p. 125); see Appendix III. This mechanism is also of special importance in lectin–carbohydrate interactions.

Thus the optimal "fit" between epitope and paratope still appears to be the most important prerequisite for the formation of a high-energy; specific Ag–Ab bond. Not only were Emil Fischer (1894) with his lock-and-key proposal, and Paul Ehrlich (1900) (for more historical details, see Silverstein, 1989) with his side-chain theory, exceedingly right in their insight in the importance of the "goodness of fit" principle, but all experimental observations reported since point to the unique importance of the best steric fit between epitope and paratope as a *conditio sine qua non* for obtaining the highest Ag–Ab-binding energy (see, e.g., Landsteiner, 1936; 1962; Pressman and Grossberg, 1968; Grossberg, 1979). A number of authors have contrasted the "lock and key" (i.e., optimal "fit") hypothesis, with a "surface accessibility" mechanism, by which mobility and deformability of the surface structures play a crucial role (see, e.g., Mariuzza et al., 1987; Davies et al., 1988; Colman, 1988; Getzoff et al., 1988; Geysen et al., 1988; Bhat et al., 1990). These two approaches however are *not* mutually exclusive; a certain degree of flexibility may be helpful in inducing a better "fit" (i.e., a lock often opens more readily when one wiggles the key). However, energetically, there is some small price to pay for pronounced surface motility: greater motility of a prominent peptide (or polysaccharide) moiety is equivalent to a (locally) enhanced Brownian movement, which (for two degrees of freedom) acts *repulsively* with an energy of +1 kT. In several specific cases, the conformational changes upon epitope–paratope binding have been reported to be fairly small (Novotny et al., 1989; see also Amit et al., 1986).

It is important to reiterate that, in biological systems, most polar compounds are *monopolar*

(i.e., they have a high $\gamma^{\ominus}$ value and a $\gamma^{\oplus}$ which is zero, or close to zero). Thus, notwithstanding the pronounced hydrophilicity (i.e., polarity) of these polar materials, their $\gamma^{AB}$ value is zero [cf. Eq. (6)]. Applying the Dupré equation for the interaction between two condensed phase materials 1 and 2 (in vacuo):

$$\Delta G_{12} = \gamma_{12} - \gamma_1 - \gamma_2 \tag{16}$$

it is clear that for monopolar materials of biological origin, $\gamma_1 = \gamma_1^{LW}$ and $\gamma_2 = \gamma_2^{LW}$. In addition in most of these cases, $\gamma_{12} \approx 0$. Therefore, after expulsion of the liquid medium, the interfacial free energy, $\Delta G_{12}$ ($= \Delta G^{LW} + \Delta G^{AB}$) $= \Delta G^{LW} \approx -\gamma_1^{LW} - \gamma_2^{LW}$. Therefore, we have the unusual situation that the following transition holds true upon the expulsion of interstitial water:

$$\Delta G_{1W2}^{IF} = (\Delta G_{1W2}^{LW} + \Delta G_{1W2}^{AB}) \xrightarrow[\text{of water}]{\text{expulsion}} \Delta G_{12}^{LW} \tag{17}$$

or, neglecting $\Delta G_{1W2}^{LW}$ as being very small:

$$\Delta G_{1W2}^{AB} \xrightarrow[\text{of water}]{\text{expulsion}} \Delta G_{12}^{LW} \tag{18}$$

In other words, an initial practically purely hydrophobic (AB) attraction, upon consolidation by the expulsion of residual bound water, becomes a pure LW bond, with an energy of the order of $-65$ to $-90$ mJ/m$^2$, which for a minimum contactable surface area ($S_c$) of 0.4 nm$^2$ corresponds to $-6.5$ to $-9$ kT. For $S_c$ values which are $x$ times higher, $x$ times stronger binding energies obtain, and $K_{ass}$ values increase to $K_{ass}^x$ (cf. Table 2).

Thus the strongest antigen–antibody bonds are essentially those in which as much as possible of the surfaces of epitope and paratope are brought closest together, i.e., with the best possible fit. The schematic presentation given in Fig. 3 of Chapter 4 is therefore only applicable as far as (primary) electrostatic multiple contact bonds are concerned. Hydrophobic bonds do not act as point contacts and direct H bonds, as discussed below, are unlikely to occur very often. The final antigen–antibody bond, after expulsion of water, bears more analogy to two adjoining closely fitting surfaces, than to irregular bodies that are welded together at a few discrete points.

## C. Short-Range Electrostatic Interactions

As a consequence of the shielding effect of the diffuse ionic double layers surrounding the charged sites (the effect of which varies strongly with the ambient ionic strength and with the distance between charged sites), the calculation of close-range EL interaction energies is a complicated operation. However, as a first approximation, the free energy ($\Delta G^{EL}$) of attraction between totally ionized $COO^-$ and $NH_2^+$ or $NH_3^+$ groups, at a distance of about 0.3 nm, in a medium with an ionic strength $\mu = 0.15$, is of the order of about $-7$ to $-10$ kcal/mol or about $-12$ to $-18$ kT for a charge interaction between an opposing epitope–paratope pair (see Tables 2 and 3). This interaction energy is of the same order of magnitude as that of a fairly typical interaction between Ag and Ab.

Electrostatic binding is, to some extent, analogous to the direct type of AB-interaction discussed below. However, with EL bonds, direct bonding is the rule, because contrary to direct H bonds (which occur only when the most precise optimal alignment between, e.g., H and O can be realized), EL attractions have the advantage of starting at a distance, so that they can grow stronger and often can automatically become more precisely aligned upon close approach.

## D. Role of Direct Hydrogen-Bonding in Specific Interactions

There is no indication from the Ag–Ab systems described in the Appendices, nor from other Ag–Ab systems which might be putative candidates for direct H-bonding mechanisms, that *direct*

H-bonding plays a significant role in Ag–Ab reactions. One reason for this is that *direct* H bonds occur only when both the precise bond angle and the exact bond distance can be achieved. The direct H bond cannot exert its attraction at distances significantly exceeding 0.2 nm and, as such, cannot *initiate* Ag–Ab interactions. Thus, the initial attraction must occur through mechanisms other than direct H-bonding.

Direct hydrogen-bonding between Ag and Ab (i.e., between OH and C=O, NH and C=O, and NH and OH groups) has been identified with certainty in only a few cases, for example, with an *o*-substituted benzoate hapten, reacting with an anti-*p*-azobenzoate Ab (Pressman and Grossberg, 1968). Neutral hydrophilic polysaccharide Ags—such as dextrans, are strong electron donors (van Oss et al., 1987; 1994), thus ruling out "hydrophobic" as well as electrostatic interactions—may conceivably interact specifically with Ab-active sites in a direct closely fitting electron-donor (Ag)–electron-acceptor (Ab) mode. However, at least on a macroscopic level, no available electron-acceptor activity of immunoglobulins has been demonstrated. Thus while direct H-bonding theoretically could exist in primary dextran–antidextran interactions,* it probably does not occur to any significant degree.† However, direct H bonds between Ag and Ab may conceivably evolve secondarily in some cases. It has been hypothesized, for example, in cases for which the primary bond occurs between a somewhat acidic Ag and a basic Ab [e.g., with DNA–anti-DNA (high-affinity Abs] that the primary electrostatic bond ultimately may evolve into a direct H bond (van Oss et al., 1985b). However, in the case of high affinity anti-DNA Abs binding to DNA, there is no energy change during the transition of primary to secondary bonding, but once the bonds have established themselves, it is no longer possible to achieve dissociation at high ionic strength (Smeenk et al., 1982). The following explanation of this appears the most reasonable one: after closest contact is made between the negative epitope and the positively charged paratope, some of the water of hydration is expelled and a largely LW interfacial bond forms between the best-fitting parts of epitope and paratope. This displaces to some degree the optimally opposing minus and plus sites on epitope and paratope, thus forming a compromise bond consisting in part of interfacial LW and in part of slightly mitigated EL bonds. Such a hybrid bond is not as easily dissociated by the admixture of salt as a simple EL bond, even though the same amount of salt (several molar) could prevent the initial bond from forming in the first place; see also Sec. XI.D on Hysteresis).



## E. Hydrogen Bonds Used in Antigen–Antibody Modeling

Whilst direct H-bonding between Ag and Ab may be an appealing idea, there are few hard experimental data to substantiate it. The practice of optimally fitting direct H bonds between epitope and paratope, among computer modelers of Ag–Ab and similar ligand–receptor interactions, may therefore have little relevance to the Ag–Ab bonds that occur in real life [see also Davies et al. (1988), who note that "not all possible H bonds are actually made"]. On the other hand, much can still be learned from the many careful experimental measurements done on a vast

*This would necessitate the unlikely scenario of three or four H bonds occurring between the nonreducing terminal glucose moiety of the dextran epitope and one amino acid, or at most two amino acids, of the paratope.

†While both "hydrophobic" interactions and direct H bonds are due to H bonds, the mechanisms in both cases are quite different. In hydrophobic interactions, the low-energy (hydrophobic) moieties are "squeezed together," through the H-bonding energy of cohesion of the surrounding water molecules (van Oss and Good, 1988; 1991). Direct H bonds, on the other hand, occur through precise C=O—HO binding between opposing sites. The similarity in the origin of both hydrophobic and direct H-bonding unfortunately makes it very difficult to distinguish between the two types of bond. For instance, any physicochemical measure resulting in the dissociation of bonds of the first type also leads to the dissociation of the second bond type.

array of actual hapten–Ab and Ag–Ab interactions, by Pressman and Grossberg (1968), and many others (see, e.g., Nisonoff et al., 1975; Kabat, 1976), all of which point to the primary importance of the optimal "fit" *between complementary shapes* of the molecules' Born–Kihara shells (van Oss and Good, 1984) (i.e, their van der Waals surfaces).

Whilst there are no strong theoretical or experimental arguments *for* a putative prevalent role of H bonds in Ag–Ab-bonding, serious doubts can be raised *against* their widespread occurrence. The two main ones are: (1) Direct H bonds are too short-range to be able to attract Ag to Ab at a distance (to surmount the normally prevailing macroscopic repulsion); and (2) direct H bonds require a very narrow range of precise bond angles, which are unlikely to occur frequently in the relatively haphazard apposition of the various amino acids (or other moieties) of epitope and paratope.* Argument (1) largely excludes direct H-bonding from playing an important role in the primary Ag–Ab interaction, while argument (2) would indicate that, although direct H-bonding in the secondary, close-range interactions between Ag and Ab could occasionally be possible, it is unlikely to be a predominant mechanism.

## IX. NONSTOICHIOMETRY OF ANTIGEN–ANTIBODY BONDS

As is obvious from the formation of soluble as well as insoluble Ag–Ab complexes, Ags and Abs (van Oss, 1979) as well as other complex-forming substances, such as cationic and anionic surfactants (van Oss, 1984), can combine in a wide range of proportions. The Ag–Ab reactions thus are essentially nonstoichiometric. As a consequence of the nonstoichiometry of Ag–Ab interactions, the determination of the valency of Ags and Abs (see below) has its own peculiar rules.

## X. VALENCY OF ANTIGENS AND ANTIBODIES

### A. Determination of Antigen and Antibody Valencies

The valency of Ags can be determined with complexes formed in an excess of Ab, while the valency of Abs can be determined only with complexes formed in an excess of Ag. However, precipitates obtained at optimal Ag/Ab ratios (van Oss, 1979) often have close to stoichiometric Ag–Ab proportions, with possibly a slight excess of Ag.

### B. Valency of Antibodies

Human antibodies of the IgG, IgA, IgD, and IgE classes are divalent; antibodies of the IgM class are decavalent (dimeric IgA, humoral or secretory, is tetravalent). IgM class Abs are especially prone to steric hindrance; that is, because of their size, large Ag molecules, when binding one of the ten paratopes of IgM, can also prevent one or several more IgM paratopes from binding other Ag molecules of the same specificity. The steric hindrance effect of IgM manifests itself at Ag molecular weights of 2000 and higher, so that with larger Ags, apparent valencies for IgM are found that are less than 10; the lowest valencies being observed with the highest molecular weight Ags (Edberg et al., 1972).

It is possible to render IgG class Abs monovalent through partial digestion with papain (such monovalent IgG molecules, called Fab fractions, lack the Fc tail; see Chap. 1).

*For instance whilst in DNA pairing vast numbers of combinations of hydrogen donors with hydrogen acceptors are *theoretically possible*, in *actual* Watson–Crick-type DNA pairing, there is an extreme restriction which allows only the double H-bonding adenine-thymine and the triple H-bonding guanine-cytosine combinations to occur.

### C. Valencies of Antigens

Most protein Ags are plurivalent only vis-à-vis a complete antiserum elicited against them containing antibodies against each of the epitopes. Each different valency site of a protein Ag usually is an epitope with a different configuration from the other valency sites. A given monoclonal Ab can react with only one valency site of such a protein Ag. Some repeating types of biopolymer may be plurivalent, with all the epitopes being identical with each other (e.g., DNA or tobacco mosaic virus; Van Regenmortel, 1982), or they may have only two or three different groups of epitopes that are identical with one another within each group (DNA also can be an example of this type of Ag). On the other hand, other repeating biopolymers may be monovalent; for example, for dextran in the ideal, totally unbranched form, the dominant epitope is the terminal nonreducing sugar (see Appendix III). The immunodominant epitopes of native globular proteins tend to be situated near their carboxyterminal and at *prominent places* on the outer periphery of their tertiary configuration (Atassi, 1984). From known valencies of globular proteins and comparable biopolymers (e.g., viruses), it can be seen that there usually is roughly one epitope for about every 35–40 amino acids. As a first approximation, one may thus estimate the valency, $N$, of a given globular protein as $N = (M/5000)^{2/3}$. The valencies ($N$) thus calculated for a number of Ags with molecular weights (M) varying from 13,000 to 41,000,000 agree well with the reported valencies (van Oss and Absolom, 1984).



## XI. ANTIGEN–ANTIBODY AND LIGAND–RECEPTOR ASSOCIATION AND DISSOCIATION

### A. Implications of the Law of Mass Action

The law of mass action, which is based on

$$Ag + Ab \leftrightarrows AgAb \tag{19}$$

and which gives rise to the expression for the association constant, $K_{ass}$:

$$K_{ass} = \frac{[AgAb]}{[Ag]\cdot[Ab]} \tag{20}$$

[see also Chap. 30, and the connection between $K_{ass}$ and the binding energy; see Eq. (14)], implies that when one changes the Ab or the Ag concentration—expressed as [Ab] and [Ag] in Eq. (20)—the amount of Ag–Ab complex that is formed will also change. For instance, if (at constant Ag concentration) one decreases the Ab concentration, the amount of Ag–Ab complex formed also will decrease, and if one increases the Ab concentration, the amount of Ag–Ab formed will increase. The same applies to other systems; one pertinent example is the reaction in vivo between the Fc moieties of IgG (Fc) and the Fc receptors (R) of phagocytic leukocytes. The binding constant, $K_{ass}$, for Fc–R is of the order of $10^6$–$10^8$ L/*M* (Froese, 1983; Dorrington and Klein, 1983). In circulating blood, phagocytic Fc receptors are bound fairly strongly to IgG, which is normally present in high concentrations, so that it is unlikely that Fc–R-mediated interactions between rather sparse sensitized particles and phagocytes takes place in the *bloodstream* to any significant degree. However, in the *spleen*, where Fc–R-mediated interactions play a more important role, the situation is different due to higher cell, and concomitantly lower IgG, concentrations as well as to high local macrophage concentrations (with high densities of receptors per cell). Thus, Fc–R-mediated interactions between sensitized cells and phagocytes are much more strongly favored in the spleen than in the vascular blood circulation (van Oss et al., 1985a). For instance, autoimmune RBC destruction by phagocytes mainly takes place in the spleen and only to a small extent in the peripheral blood circulation. In the same manner, it

becomes clear why *depleted* blood IgG levels (achieved, e.g., by absorption or peripheral IgG by protein A columns) can have therapeutic antitumor effects in vivo (Sjögren et al., 1982), as that treatment causes an increase in free receptors, which favors their interaction with specific antitumor antibodies of the IgG3 subclass, which are not removed by protein A and have the strongest affinity to receptors of the four IgG subclasses. It also is easy to understand how massive *increases* in circulating IgG, brought about by multiple transfusions of gamma globulin solutions, causes a significant (but usually temporary) amelioration in the clinical course of autoimmune thrombocytopenic purpura and in autoimmune neutropenia, because the sensitized platelets or neutrophils of these patients are less readily destroyed by their phagocytic cells as long as the phagocytes' receptors are swamped by a large increase in circulating IgG (van Oss et al., 1985a). It also becomes clear why the interaction in vitro of $Rh_o$(D)-positive human RBCs sensitized with IgG class anti-$Rh_o$(D) antibodies, with the Fc–R of monocytes, strongly decreases in the presence of increased concentrations of ambient IgG. It is also obvious from Eq. (20) that simple centrifugal washing of phagocytes will more strongly favor detachment of single immunoglobulin molecules the lower the value of $K_{ass}$. At very high $K_{ass}$ values (of the order of $10^{10}$), removal of immunoglobulin (e.g., IgE) by washing becomes virtually impossible. Measurement of [Fc] and [Fc-R] done at different dilutions can yield values for $K_{ass}$ as well as for the total number of receptors per cell, even if [R] is unknown (van Oss et al., 1985a). It should be emphasized that the foregoing considerations are as applicable to Ag–Ab interactions as to other ligand–receptor interactions.



## B. Other Effects of Dilution on Antigens and Antibodies

Contrary to a widely held belief, the binding constant, $K_{ass}$ as measured by the usual methods, is *not* an invariable parameter which is uniquely characteristic of a given Ag–Ab reaction. For example, at optimal Ag–Ab ratios, $K_{ass}$ of the BSA–anti-BSA reaction increases from $1.7 \times 10^7$ L/*M* to $6.5 \times 10^{11}$ L/*M* on 100-fold dilution of both reagents (van Oss and Walker, 1987). While this increase is undoubtedly due in part to the polyclonality of the Ab (where, of course, at greater dilution the higher-affinity Abs are the principal Abs that still bind), this trend will persist even with monoclonal Abs. Thus, it should be faced that many, if not most, of the published $K_{ass}$ values for various Ag–Ab (or Ig–receptor) interactions reflect the *dilution* at which the measurements were done more strongly than the actual unique binding constant that could be believed to be a characteristic property of the system under study. For instance, the high $K_{ass}$ values published for the binding of IgE to Fc receptors on basophils and mast cells (Froese, 1983) is as likely to be a function of the very low IgE concentrations which one is normally forced to use, as of an intrinsically high $K_{ass}$. The best solution for obtaining reasonably reliable $K_{ass}$ values, regardless of the strong influence of the volume in which the reaction occurs, is to follow the rules given by Van Regenmortel and Hardie (1979), which include operating under constant conditions of the percentage of occupied binding sites (e.g., 50% binding sites occupied) *and* measuring at a wide range of reagent concentrations.

Dilution favors Ag–Ab dissociation, especially of the lower-affinity components, although $K_{ass}$ may increase as dilution favors the higher-affinity Abs (see above). An electric field (electrophoresis) applied to, for example, Ag–Ab complexes associated with an Ab fixed to an immobilized carrier, will favor dissociation when the Ag can be electrophoretically removed from (and prevented from returning to) the fixed Ab. In such cases, obeying the law of mass action [see Eqs. (19) and (20)], continuous removal of one of the reagents (Ag) by electrophoresis (i.e., through artificial dilution of one of the participants) displaces the equilibrium described in Eq. (19) to the left, leading to dissociation.

## C. Affinities of Monoclonal and Polyclonal Antibodies

The average affinity of monoclonal Ab mixtures (MAbs) to a given epitope is generally lower than that of polyclonal Abs (PAbs) to the same epitope. A lower $K_{ass}$ value of MAbs to multivalent Ags (comprising several *different* epitopes) is to be expected because of the lack of cooperative cross-linking that would otherwise occur with the help of the different paratopes of PAbs. This drawback of MAbs can be surmounted by using mixtures of two or more MAbs that are specific for different epitopes of the same Ag. Single MAbs normally cannot form immune precipitates with Ags, but immunoprecipitation does become possible with mixtures of two or more MAbs (Molinaro et al., 1984). Single MAbs, however, can form immune precipitates with large Ags (e.g., plant viruses) with repeating identical epitopes (Halk et al., 1984). In these cases, however, the distinction between immune precipitation and agglutination becomes hazy and, clearly, hemagglutination with MAbs is entirely feasible.

The apparent lower affinity (expressed as $K_{ass}$) of MAbs, in comparison with PAbs, is mainly because when measuring $K_{ass}$ of PAbs, one actually measures mainly the $K_{ass}$ of the PAb components with the highest affinity, even though such high-affinity PAb components represent only a small fraction of the total. In other words, high-affinity PAb *components* represent a small minority, which is one of the reasons why high-affinity MAbs are rare. Bankert et al. (1981) observed that various MAbs directed against 4-azophthalate have a fairly wide array of binding constants (ranging from $4 \times 10^4$ to $4 \times 10^7$), the highest value of which compares well with the higher values obtained with polyclonal Ags against haptens (Pfeiffer et al., 1984).



## D. Primary and Secondary Bonds: Hysteresis

### 1. Primary Bonds

The specificity of the bonds between epitope and paratope (and between ligands and receptors in general) is principally due to the *close-range* interactions that occur very early during the Ag–Ab binding process. However, although the specificity of Ag–Ab interactions is mainly linked to the primary bond, in many cases, the primary bond energy is significantly smaller than the total (primary plus secondary) bond energy (van Oss et al., 1979; 1982; 1986b; Absolom and van Oss, 1986).

The primary bond energy can be determined by measuring the energy required to prevent the bond from forming; see Eq. 21. A few examples of primary bonds can be given. First, to prevent bovine serum albumin (BSA) from reacting with anti-BSA, it suffices to raise the pH from 7.0 to 9.5 (van Oss et al., 1982). However, to *dissociate* BSA–anti-BSA complexes, once formed, it is necessary to raise the pH to 9.5 *and* to add 9.7 *M* ethylene glycol (van Oss et al., 1979). In a purely polar system, to prevent the interaction between 3-azopyridine (P3), coupled to rabbit serum albumin, with rabbit anti-P3, it suffices to add 1.9 *M* dimethyl sulfoxide (DMSO), but the energy needed to *dissociate* anti-P3 from P3 requires the admixture of 6.4 *M* DMSO (van Oss et al., 1979). In mainly electrostatic systems, on the other hand (e.g., low- and medium-affinity DNA–anti-DNA systems; Smeenk et al., 1982), no difference could be measured between the primary and the total energy of association, as deduced from the equality between the energies of dissociation and of prevention of association.

Whilst primary bonds of the sole Lifshitz–van der Waals variety are virtually nonexistent,*

*In this context, LW bonds which occur after expulsion of water of hydration (see above: The $\Delta G_{1W2} \rightarrow \Delta G_{12}$ transition) are considered to be secondary bonds.

instances for which the other bond types occur as the sole primary bond are common: (1) primary polar (or "hydrophobic") bonds: P3–anti-P3 (the hapten 3-azopyridine) and dextran–antidextran (see Appendices I and III), and (2) primary electrostatic bonds: BSA–anti-BSA (see Appendix IV) and idiotype–anti-idiotype (Absolom and van Oss, 1986).

## 2. Secondary Bonds

*Hysteresis.* The much greater energy usually needed for dissociating most Ag–Ab bonds than is required for the prevention of their association (*hysteresis*) is due to the existence of further secondary bonds that have formed subsequent to the formation of the initial primary Ag–Ab bonds. The energy of the secondary bond ($\Delta G_{sec}$) is obtained as follows:

$$\Delta G_{sec} = \Delta G_{dissociation} - \Delta G_{prevention\ of\ association} = \Delta G_{total} - \Delta G_{primary} \tag{21}$$

Secondary bond formation is closely linked to the time factor discussed by Van Regenmortel (1989).

Electrostatic bonds rarely occur as secondary bonds. The purely electrostatic DNA–anti-DNA system has been studied from this aspect: The similarities of pH conditions, leading to dissociation or to prevention of association (lack of hysteresis), points to an absence of secondary electrostatic bonds in this system (see above; Smeenk et al., 1982). Secondary electrostatic bonds are likely to be rare occurrences, as the probability that negatively and positively charged amino acids on Ag and Ab (outside of the epitope and paratope) are situated precisely opposite each other is very slight and, in those rare cases when it might occur, such moieties would be indistinguishable from the epitope and paratope, and such an Ag–Ab system simply would behave as a system with somewhat larger than usual epitopes and paratopes.

Polar (AB or "hydrophobic") interactions are the most common bonds involved in secondary Ag–Ab bonding. As soon as epitope and paratope have combined in a primary bond, various nonspecific (especially nonpolar) moieties of Ag or Ab in the immediate vicinity of epitope and paratope, which can undergo a "hydrophobic" attraction, approach each other more closely, and then bind to each other secondarily. Thus, both when the primary bond is mainly "hydrophobic" (e.g., P3–anti-P3) *and* when the primary bond is largely electrostatic (BSA–anti-BSA), secondary bonds of the (AB) "hydrophobic" type almost invariably develop.

With time, a further strengthening of existing (primary as well as secondary) bonds of all categories takes place through the extrusion of interstitial solvent. This results in a shorter distance between epitope and paratope, which considerably enhances the interfacial attraction energy, which is almost exclusively of the LW variety (cf, the earlier Sec. VIII).

# E. Conditions Favoring Antigen–Antibody Association or Dissociation

The conditions favoring association of Ag with Ab usually are, at least qualitatively, the inverse of the conditions favoring their dissociation. For the sake of simplicity, it suffices to describe only the various conditions under which dissociation of Ag–Ab complexes can be achieved.

Dissociation usually is most readily achieved by *combining* the admixture of a strong electron-donor organic solvent (e.g., dimethylsulfoxide, ethylene glycol, propanol) to the liquid medium, with an increase (or occasionally with a drastic decrease) in pH (van Oss et al., 1979; 1986b; Absolom and van Oss, 1986). Only relatively weak, purely electrostatic systems dissociate well by just increasing the ionic strength; in systems with mainly "hydrophobic" bonding, however, raising the ionic strength tends to be counterproductive (because high salt concentrations cause dehydration which, in its turn, causes a further increase in surface "hydrophobicity").

Addition of chaotropic salts, on the other hand, favors dissociation of both "hydrophobic" and electrostatic bonds because they have the capacity of increasing the ionic strength as well as of opening up, or of displacing, hydrogen bonds, and thereby of dissociating bonds due to "hydrophobic" interactions (Absolom and van Oss, 1986).

Elution of blood group Abs from RBCs (anti-A, anti-K, anti-D) using the combined DMSO and increased pH approach has been described by van Oss et al. (1981), who also reviewed some other blood group Ab elution methods. Helmerhorst et al. (1982) described the efficiency of various Ab elution approaches (including elution with DMSO *and* pH 9.5; elution at pH 2.8; elution by heating at 56°C; elution with ether), for the elution of granulocyte, platelet, and HLA Abs. A large number of conditions favoring Ag–Ab dissociation as well as the prevention of Ag–Ab formation have been listed by Absolom and van Oss (1986), including several blood group Ag–Ab systems.

Exclusively electrostatic bonds (when relatively weak) are the easiest to dissociate by mild increases or decreases in pH or by the admixture of NaCl. Examples of these are DNA–anti-DNA complexes of medium or low affinity (De Groot et al., 1980; Smeenk et al., 1982; van Oss et al., 1985b). Higher-affinity DNA–anti-DNA complexes can be dissociated at pH $\approx$ 12, but not by the admixture of salt (De Groot et al., 1980). Another system of this type is that of human idiotype–anti-idiotype complexes, which are the cause of the occurrence of IgG dimers and oligomers in pooled human plasma gamma globulin fractions. Almost all of these complexes dissociate at pH 4 and about one-third of the complexes dissociate in 4 *M* NaCl (Tankersley et al., 1988).



## F. Effect of Temperature

It has already been shown that an increase in temperature often causes no significant change in $\Delta G$ owing to the enthalpy–entropy compensatory effect discussed earlier (cf., Sec. VIII). However, other factors remaining equal, it should be remembered that a small decrease in $\Delta G^{TOT}/kT$ due to an increase in temperature can result in a more pronounced decrease in $K_{ass}$ [see Eq. (14)]. For instance, an increase in temperature from 20° to 50°C, for an initial value of $\Delta G^{TOT}$ of $-16$ kT (which then becomes reduced to $-14.5$ kT), results in a 4.4-fold decrease in $K_{ass}$ from $8.9 \times 10^6$ L/*M* to $2.0 \times 10^6$ L/*M*. An increase in temperature also causes a decrease in the energy of hydrophobic attraction, as a consequence of the fact that at higher temperatures water becomes less Lewis basic and more Lewis acidic (van Oss, 1994a). In Ag–Ab reactions, however, other factors do not necessarily remain equal when the temperature increases (e.g., hydration decreases, the distance between Ag and Ab decreases, but H-bonding and LW energies also decrease). However, about 30% of the cases, it was possible to elute granulocyte or platelet Abs by heating to 56°C for 60 min (Helmerhorst et al., 1982).

## G. Effect of Other Factors

### 1. Time

With the lapse of time, the Ag–Ab bond becomes stronger as a consequence of secondary interactions. Thus, if one aims at *dissociation* (e.g., with a view to an affinity separation step), it is advantageous to effect the dissociation procedure *as soon as possible* after the Ag–Ab bonding has taken place, cf. Van Regenmortel (1989).

### 2. Haptens

The admixture of haptens (Hp), which are identical to the epitope of an Ag in a given Ag–Ab system, will cause a dissociation of Ag–Ab in favor of the association of Hp–Ab. This phenomenon can be used to advantage, especially in dissociating lectin–blood group epitope bonds by means of the addition of the lectin-specific sugar.

### 3. Influence of Strong Electron-Donor Polymers

Strong electron-donor polymers, such as polyethylene glycol (PEG) at concentrations of about 3–10% (v/v) will repel most other polymers in aqueous solutions (e.g., serum proteins). This causes an incipient phase separation, which then pushes the proteins into a smaller volume and also partly dehydrates them. This effect favors the formation of Ag–Ab complexes. However, care must be taken not to overdo the effect, because free immunoglobulins will start to precipitate at only slightly higher PEG concentrations than needed for enhanced Ag–Ab association.

### 4. Influence of Strong Dehydrating Agents

The addition of $(NH_4)_2SO_4$ in molar concentrations will favor the insolubilization of Ag–Ab complexes through dehydration, thereby enhancing the hydrophobic (AB) attraction and decreasing the distance between epitope and paratope (as also occurs with PEG; see above). However, when the Ag–Ab system is mainly electrostatic, $(NH_4)_2SO_4$ on the contrary, will tend to *dissociate* such complexes (cf., Sec. XI.D.2).

## XII. SUMMARY OF THE MECHANISMS OF SPECIFIC LIGAND-RECEPTOR (e.g., ANTIGEN–ANTIBODY) INTERACTIONS

The following steps are involved in specific ligand–receptor interactions, taking Ag–Ab interactions as an example (and keeping in mind that the Ag-active site is the *epitope* and the Ab-active site the *paratope*):

### A. Primary Interaction

The normal (mainly AB) long-range ($\ell \geq 5$ nm) macroscopic repulsion between Ag and Ab must locally be overcome by a medium to short-range epitope–paratope attraction (see Fig. 1). This is achieved through the placement of the epitopes (and especially the immunodominant epitope) on prominent, protruding parts of the Ag molecule or particle, with a small radius of curvature and an overall small total surface area of 0.4–10 nm$^2$. The primary bond energy, which may be expressed as $\Delta G_{1W2}$, is significantly attractive at a distance of 2–3 nm. Primary bond energies can be of the LW + AB, or of the LW + EL + AB classes (the LW interaction generally is by far the feeblest). The LW, AB, and EL attractive energies decay with distance according to different regimens and, therefore, must be calculated separately.

Direct H bonds play no role in the primary interaction.

### B. Secondary Interaction

Once the first (primary) contact is made between epitope and paratope, two things happen:

1. After the first contact between the (still hydrated) epitope and paratope, their water molecules of hydration become expelled into the bulk liquid, thereby increasing the entropy of the system, because previously oriented water molecules of hydration become randomized upon expulsion. If the fit between epitope and paratope is optimal, the final dehydration step gives rise to a switch in free-energy designation from $\Delta G_{1W2}$ to $\Delta G_{1W}$, which among biopolymers generally entails a large increase in bond energy, allowing sizable attractive energies to occur with small epitope–paratope interfacial areas. Whilst $\Delta G_{1W2}$ usually is mainly represented by $\Delta G^{AB}_{1W2}$, $\Delta G_{12}$ comprises mainly LW interactions. Upon the mutual neutralization of oppositely charged sites on epitope and paratope, a further strengthening of the EL bond also occurs after expulsion of water.

2. Between the Ag and Ab chains immediately neighboring the epitope and paratope, which are brought closely together through the primary attraction, secondary largely nonspecific bonds of the LW and AB variety tend to form, which further increase the overall bond energy. Direct H bonds rarely play a role in secondary interactions.

It is only when the epitope fits the paratope precisely that all interstitial water of hydration is expelled, so that everywhere at the interface, $\ell = \ell_0$, which favors the strongest possible bond. The principal prerequisite for a strong Ag–Ab (or other ligand–receptor) bond is, therefore, the optimal fit between the Born-Kihara shells of epitope and paratope (also often alluded to as their van der Waals surfaces), regardless of their mode(s) of interfacial interaction. In general, it is advisable to treat all specific and aspecific non-covalent interfacial bonds as interactions between surfaces with a well-defined area, rather than as interactions between point sites, with the possible exception of EL interactions.

## LIST OF ABBREVIATIONS, SYMBOLS, AND UNITS

### Antigens and Antibodies

| | |
|---|---|
| Ab | Antibody |
| Ag | Antigen |
| Ag–Ab | Antigen-antibody, or antigen-antibody complex(es) |
| MAb | Monoclonal antibody |
| EPI (subscript) | Epitope, or antigenic determinant |
| PARA (subscript) | Paratope, or antibody-active site |
| Ig | Immunoglobulin(s): IgA, IgD, IgE, IgG, IgM |
| Fc | Carboxyterminal Ig moiety |
| P3 | 3-azopyridine (hapten) |

### Length

| | |
|---|---|
| Å | Ångström = $10^{-8}$ cm = 0.1 nm |
| $\ell$ | Interparticle or intermolecular distance |
| $\ell_0$ | Distance at the minimum equilibrium distance between particles or macromolecules; $\ell_0 \approx 1.5$ to 1.6 Å |
| R | Radius of spheres (also: receptor; gas constant) |

### Superscripts and Subscripts

| | |
|---|---|
| *LW* (superscript) | Lifshitz-van der Waals |
| *AB* (superscript) | (Lewis) acid–base, or pertaining to electron acceptor/electron donor interactions; in aqueous systems, also pertaining to Brønsted acid–base interactions |
| *EL* (superscript) | Electrostatic (or electrokinetic) |
| *BR* (superscript) | Brownian movement |
| *TOT* (superscript) | Total |
| *L* (subscript) | Liquid |
| *S* (subscript) | Solid |
| *i*, *j*, *w* (subscripts) | Generalization for subscripts, 1, 2, 3, *w* |

## Free Energy, Surface Tension, and Interfacial Tension

| | |
|---|---|
| $\Delta G$ | Free energy of interaction: expressed in energy units (per particle, per mole, per molecule, or per unit surface area |
| $\Delta G_{132}$, $\Delta G_{1W2}$ | Free energy change of interaction between materials 1 and 2, immersed in liquid 3 or in water (units: J, erg, kcal, kT [= $4.04 \times 10^{-21}$ J at 20°C]) |
| $\Delta G_{131}$, $\Delta G_{1W1}$ | Free energy change of interaction between two particles or molecules of material 1, immersed in liquid 3 or in water (units as above) |
| $\Delta H$ | Enthalpy, i.e., heat change component of $\Delta G$ (units as above) |
| $\Delta S$ | Entropy, i.e., "disorder" change component of $\Delta G$ (units: calories per mole degree) |
| $\gamma_i$ | Surface tension of liquids and solids |
| $\gamma_{ij}$ | Interfacial tension between two liquids or between a liquid and a solid or solute |
| $\gamma^{\oplus}$ | Surface tension: electron acceptor parameter |
| $\gamma^{\ominus}$ | Surface tension: electron donor parameter |
| $\gamma^{LW}$ | Lifshitz-van der Waals component of the surface tension |
| $\gamma^{AB}$ | (Lewis) acid-base component of the surface tension |
| | All surface tensions, their components, and parameters may be expressed in units of tension (e.g., $mN/m^2$) or, preferably, in units of energy per unit surface area (e.g., $mJ/m^2$, $ergs/cm^2$), to bring them on a level comparable to that of $\Delta G$; cf, Eq. 2. |

## Energy Units and Related Symbols

| | |
|---|---|
| J | Unit of energy = $10^7$ ergs = 0.239 cal |
| kcal | Kilocalorie = $4.186 \times 10^{10}$ ergs = 4186 J |
| $mJ/m^2$ | (= $erg/cm^2$). The $mJ/m^2$ is the preferred unit of surface tension ($\gamma_1$), interfacial tension ($\gamma_{ij}$), and (often) of interfacial free energy ($\Delta G_{132}$, $\Delta G_{131}$) |
| $kT$ | Unit of energy per particle, cell, or molecule (1 kT = $4.04 \times 10^{-21}$ J at 20°C, or 293° Kelvin) |
| $k$ | Boltzmann's constant (k = $1.38 \times 10^{-23}$ J per degree Kelvin, per molecule or particle) |
| $R$ | Gas constant ($R = 1.986 \times 10^{-3}$ K cal = $8.31 \times 10^{10}$ ergs = 8.31 J, per degree Kelvin per mole); $R = 6.022 \times 10^{23}$ k, where $6.022 \times 10^{23}$ equals the number of molecules in 1 mole |
| $T$ | Absolute temperature (in degrees Kelvin) |

## Law of Mass Action

| | |
|---|---|
| $K_{ass}$ | Association constant (in liters per mole) |
| $K_d$ | Dissociation constant (in moles per liter) |
| $k_{1,2}$ | Kinetic association constant (in liters per mole/sec) |
| $k_{2,1}$ | Kinetic dissociation constant (in $sec^{-1}$) |
| $M$ | Mole, or molar |

## Other Physical Units, Entities, or Symbols

| | |
|---|---|
| A | Hamaker, or van der Waals constant, usually in $10^{-21}$ J, or in $10^{-14}$ ergs |
| C | Concentration (usually in moles per liter) |

| | |
|---|---|
| D | Diffusion coefficient |
| L | Liter |
| Mw | Molecular weight |
| $R$ | Receptor |
| $S_c$ | Contactable surface area, between Ag and Ab, or between any two macromolecules |
| $c$ | Charge of the electron ($c = 4.8 \times 10^{-10}$ electrostatic units) |
| mV | Millivolts |
| $n_i$ | Number of ions of species, $i$, per cubic centimeter |
| $v_i$ | Valency of ionic species, $i$, in solution |
| • | Dielectric constant (for water at 20°C, • = 80) |
| θ | Contact angle (in degrees) |
| ζ | (Electrical) zeta-potential of particles measured at the slipping plane (in millivolts) |
| κ | Inverse Debye length, or inverse of the thickness of the diffuse ionic double layer |
| $\psi_0$ | (Electrical) psi-nought potential at the exact interface between particle and liquid |



## XIII. APPENDICES: SURFACE THERMODYNAMIC BOND ANALYSES

### Appendix I. Dextran–Anti-Dextran, a Mainly Lifshitz–van der Waals and Partly Acid–Base-Specific Interaction

In certain cases it is possible to estimate the total interfacial (*IF*) contribution to a specific interaction ($IF = LW + AB$). The interfacial interaction energy can be determined (e.g., in cases where either ligand or receptor is known to have no, or only a negligible electrostatic potential, and for which the surface properties of the active sites can be estimated). An example of such a case is the dextran–anti-dextran system. Dextran is one of the least-charged biopolymers: its ζ-potential is −0.05 mV at ionic strength 0.15, and −0.55 mV at ionic strength 0.015 (van Oss et al., 1974). In practice, ζ-potentials smaller than ≈ 10 mV have no measurable influence on intermolecular or interparticle interactions. Thus, electrostatic interactions play no role in this system. The association constant found in the dextran (rabbit IgG)–anti-dextran system (Edberg et al., 1972; Kabat, 1976, p. 126) is $K_{ass} \approx 10^5$ L/mol. Thus, according to Eq. (14), $\Delta G^{IF}_{1W2} = -7.5$ kT, see Table 2. If we make the assumption that the paratopic cleft in the IgG (anti-dextran) molecule has the approximate "hydrophobicity" of dry IgG, at pH 7, with $\gamma^{LW}_{IgG} = 42$ mJ/m$^2$, $\gamma^{\oplus}_{IgG} = 0.3$ mJ/m$^2$ and $\gamma^{\ominus}_{IgG} = 8.7$ mJ/m$^2$, and for dextran (van Oss, 1994), $\gamma^{LW}_{DEX} = 41.8$ mJ/m$^2$, $\gamma^{\oplus}_{DEX} = 1$ mJ/m$^2$, and $\gamma^{\ominus}_{DEX} = 47.2$ mJ/m$^2$, then [cf. Eq. (8)], $\Delta G^{IF}_{1W2} = -7.13$ mJ/m$^2$, so that in units of kT [assuming the contactable surface area, $S_c$, of the paratopic cleft to be about 4 nm$^2$ for an α-(1→6)-linked hexamer (see, e.g., Kabat, 1976, p. 124)], $\Delta G_{1W2} = -7.1$ kT per paratope–dextran epitope pair (cf. Table 2). This correlates reasonably well with the value of −7.5 kT found via a measured association constant of $10^5$ L/mol. There thus does not appear to be significant scope for the formation of a further, secondary, *direct* hydrogen bond to occur between one of the glucose moieties of the isomaltohexaose epitope and a paratopic amino acid. It is important to note that there is a significant interfacial primary *attraction* between epitope and paratope. In this case the attraction ($\Delta G^{IF}_{1W2}$) is 91% due to LW forces, dextran being so hydrophilic that the AB contribution to its attraction to a moderately "hydrophobic" surface is relatively small. Thus,

while there is a sizeable close-range attraction, the attraction at a distance is fairly slight: at $\ell = 1.9$ nm it is already down to about one kT unit.

## Appendix II. Antibody-Binding to the Nitrobenzene Epitope: A Mainly Acid–Base and Partly Lifshitz–van der Waals-Specific Interaction

Another purely interfacial-specific interaction occurs between the electrically neutral hapten, or (when bound to a carrier macromolecule) the epitope, dinitrophenol (DNP), which has been widely employed in studies on hapten–antibody interactions (Kabat, 1968; 1976; Pressman and Grossberg, 1968; Turk and Parker, 1977). In the DNP epitope, it is the (uncharged) dinitrobenzene part of the molecule that is important in the specific bond with the Ab (Pressman and Grossberg, 1968). A simpler epitope of the same class, which has also been much studied, is the nitrobenzene (NB) group (Pressman and Grossberg, 1968); this epitope can be scrutinized more closely, as the surface thermodynamic data of nitrobenzene are known ($\gamma_1^{LW} = 41.3$; $\gamma_1^{\oplus} = 0.26$; and $\gamma_1^{\ominus} = 6.6$ mJ/m$^2$; van Oss, 1994a). Again, assuming that the surface of the paratopic cleft in the IgG (anti-NB) molecule has the approximate surface properties of dry IgG, at pH 7, with $\gamma_2^{LW} = 42$, $\gamma_2^{\oplus}$ 0.3, and $\gamma_2^{\ominus} = 8.7$ mJ/m$^2$, then [cf. Eq. (8)] $\Delta G_{1W2}^{LW} = -6.4$, and $\Delta G_{1W2}^{LW} = -41.4$ mJ/m$^2$, so that $\Delta G_{1W2}^{IF}$ −47.8 mJ/m$^2$. The AB attraction thus represents ≈87% of the total in this system. From the contactable surface area of NB (from its solubility in water and its $\gamma_{1W}$ value), $S_c = 0.68$ nm$^2$, one finds that a $\Delta G_{1W2}^{IF}$ of −47.8 mJ/m$^2$ corresponds to −8.0 kT. Without the $NO_2$ group (i.e., for the interaction of the benzene group with the hydrophobic IgG cleft), one finds $\Delta G_{1W2}^{IF} = -59.0$ mJ/m$^2$ which, for the $S_c$ value of benzene of 0.215 nm$^2$, means $\Delta G_{1W2}^{IF} = -3.1$ kT. Now, according to Pressman and Grossberg (1968, p. 66), the presence of the $NO_2$ group strengthens the bond by at least 2.5 kcal/mol (i.e., by at least 4.3 kT) yielding $-3.1 - 4.3 = -7.4$ kT, which is close to, but slightly lower than the value of −8.0 kT for the nitrobenzene–IgG–cleft interaction found above. Thus here also, if there is any additional, direct, secondary hydrogen bond formation between the $NO_2$ group and an amino acid of the paratope, it is unlikely to be very significant.

However, in contrast with the dextran–anti-dextran interaction discussed earlier, the nitrobenzene–anti-nitrobenzene interaction is, because of the much more pronounced "hydrophobicity" of nitrobenzene, strongly attractive at a distance. For a decay length of water, applicable to "hydrophobic" AB attractions, of $\lambda \approx 10$ nm (Christenson, 1988; van Oss, 1994a), even at a distance of ≈25 nm, $\Delta G_{1W2}^{IF}$ still has a value of about −1 kT. This may also be one of the reasons why, contrast with dextran, the nitrobenzene (or nitrophenyl) group is so strongly immunogenic.

## Appendix III. Antibody-Binding to the Nonreducing Glucose Terminal of Dextran: A Direct Interfacial Interaction Consolidated by the Expulsion of Water

With dextran as well as with other polysaccharides (branched or otherwise), the terminal nonreducing sugar residue is the most important immunodominant moiety (Kabat, 1976, p. 126). The nonreducing half of glucose is probably the "primary point of attachment" to the epitope (Kabat, 1976, p. 125). It also is the less hydrophilic (or the more "hydrophobic") moiety of the terminal glucose molecule, as well as somewhat less hydrophilic than its polymer, dextran (van Oss, 1994a). At contact ($\ell = \ell_0$), $\Delta G_{1W2}$ between a glucose molecule and its IgG paratope* equals

*For glucose: $\gamma_1^{LW} = 42.2$; $\gamma_1^{\oplus} = 0$; and $\gamma_1^{\ominus} = 34.4$ mJ/m$^2$; and for the IgG paratope: $\gamma_2^{LW} = 42.0$; $\gamma_2^{\oplus} = 0.3$; and $\gamma_2^{\ominus} = 8.7$ mJ/m$^2$ (van Oss, 1994a).

−2 kT (of which one-third is due to LW and two-thirds to AB forces), but at a distance of only 0.35 nm (equal to one intervening layer of water of hydration), $\Delta G_{1W1}$ ($\ell$ = 0.35 nm) = − 0.73 kT. Thus, $\Delta G_{1W2}$ barely suffices to provide the primary moderately long-range attraction needed to lure the immunodominant epitope to the paratope. However, the exceptional smallness of this epitope and the fact that it is located at the end of a long, thin polymer strand, easily enables it to pierce the repulsion field. Also, once on or near the paratope, the nonreducing glucose moiety (which can account for 40% of the total dextran–anti-dextran bond according to Kabat, 1976, p. 125), upon losing all the interstitial water of hydration, can bind to the paratope with a final bond energy, $\Delta G_{12}$ = −90.6 mJ/m² [see Eq. (16)]. It should be noted that in cases like these, $\Delta G_{12}$ mainly comprises LW contributions. For a contactable surface area, $S_c$, of 0.2 nm² for the nonreducing moiety of the glucose molecule, (where the entire glucose molecule has an $S_c$ value of 0.4 nm²), this corresponds to $\Delta G_{12}$ = −4.5 kT, or −2.6 kcal/mol, which correlates well with the contribution of the immunodominant glucose moiety of −2.9 kcal/mol given by Kabat (1976, p. 125).

## Appendix IV. Antibody-Binding to Bovine Serum Albumin: A Specific Electrostatic Interaction

The relatively large antigen, bovine serum albumin (BSA), which has six immunodominant surface epitopes (Atassi, 1984) is specifically attracted to the anti-BSA Ab primarily through EL forces. Epitopes 1–3 (and especially 1 and 2) show a rather close resemblance; among other points of similarity, each has three neighboring basic amino acids, which would tend to be attracted to acidic amino acids in the paratope. This agrees well with the fact that whilst BSA binds strongly to anti-BSA Abs at neutral pH, at pH 9.5 virtually no reaction between BSA and anti-BSA Abs can be observed (van Oss et al., 1982). The *primary* specific attraction, therefore, is clearly electrostatic: at pH 9.5 the basic epitope moieties, which consist of Arg—Arg—His (1 and 2) and of Lys—His—Lys (3) (Atassi, 1984), lose most (if not all) of their dissociated basicity. Three neighboring positive charges in various stages of dissociation at pH 7, attracting negative charges of the paratope, would correspond fairly closely to the interaction between *one* totally dissociated $NH_3^+$ reaction with an equally dissociated $COO^-$, so that the total interaction energy, $\Delta G_{12}^{EL} \approx -10.2$ to −10.7 kcal/mol (van Oss et al., 1982), agrees well with the value of $\Delta G_{12}^{EL}$ ≈ −10kcal/mol given by Gabler (1978) for such a case. However, once BSA–anti-BSA complexes have been formed at neutral pH, increasing the pH to 9.5 is to no avail for dissociating them. Thus, other, nonelectrostatic, secondary bonds form, once contact is made through EL interactions, which are impervious to pH changes. These are due in part to the "hydrophobic" attraction between the paratope and the more apolar peptide moieties of the epitopes: Leu—Tyr—Glu—Ile—Ala (epitope 1), Leu—Tyr—Glu—Tyr—Ser (epitope 2), and Leu—Val—Glu—Leu—Leu (epitope 3) (Atassi, 1984). Further contributions to the secondary, "hydrophobic" (AB) attraction, are furnished by the interactions between chains close to the epitope and paratope chains which are brought more closely together through the primary reaction.

## NOTE ADDED IN PROOF

Up to the present, $K_{ass}$ has generally been expressed in units of L/mol in equations of the type of Eq. 14, to obtain $\Delta G$, usually in units of kcal/mol. Strictly speaking, however, this is not correct. When using Eq. 14, it is a thermodynamic requisite to express $K_{ass}$ in dimensionless units, i.e., in (inverse) mol fractions (see Table 2 and footnote e thereof). This by no means implies that one should cease to express the equilibrium binding constant $K_{ass}$ (Eq. 20) as L/mol, as a perfectly

legitimate measure of binding affinity or avidity (see, e.g., Karush, 1976). It only means that *for the purpose of converting $K_{ass}$ to S.I. units of energy* ($\Delta G$ in Eq. 14), $K_{ass}$ needs to be in the form of (inverse) mol fractions. Thus, whilst $K_{ass} = 1.2 \times 10^6$ (mol fraction)$^{-1}$ yields $-14$ kT, or $-8.15$ kcal/mol (see Table 2), this energy level corresponds to a binding constant of only $1.2 \times 10^6/55.56 = 2.16 \times 10^4$ L/mol.

It should be noted that, e.g., W. Kauzmann (*Adv. Protein Chem., 14*: 1, 1959) advocated the use of mol fractions, while Pressman and Grossberg, 1968 consistently used mol fractions, in the guise of $K_{rel}$.

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