

## Studying Receptor-mediated cell adhesion at the single molecule level

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**Summary.** Cell adhesion is essentially mediated by specific interactions between membrane receptors and ligands. It is now apparent that the mere knowledge of the on- and off-rate of association of soluble forms of these receptors and ligands is not sufficient to yield accurate prediction of cell adhesive behavior. During the last few years, a variety of complementary techniques relying on the use of hydrodynamic flow, atomic force microscopy, surface forces apparatus or soft vesicles yielded accurate information on i) the dependence of the lifetime of individual bonds on applied forces and ii) the distance dependence of the association rate of bound receptors and ligands. The purpose of this review is, first to recall the physical significance of these parameters, and second to describe newly obtained results. It is emphasized that molecular size and flexibility may be a major determinant of the efficiency of receptor mediated adhesion, and this cannot be studied by conventional methods dealing with soluble molecules.

**Keywords.** Cell Adhesion, Intermolecular forces, Flow chamber, Surface Forces Apparatus, Atomic Force Microscopy, Biomembrane probe.

### INTRODUCTION

Cell adhesion is usually mediated by specific interactions between dedicated membrane receptors and ligands. The study of numerous biological systems revealed that the outcome of intercellular contacts is not entirely accounted for by the affinity between interacting adhesion molecules. Here are a few representative examples :

Inflammation is a ubiquitous process of prominent pathological significance. An early step is the initial interaction between flowing leukocytes and activated endothelial cells expressing adhesion molecules called selectins. These molecules have a unique capacity to bind cells passing by with a velocity of several hundreds of micrometers per second and make them roll on the vessel wall with a hundredfold slower pace. An attractive hypothesis would be that the functional properties of selectins be linked to i) particularly high rates of association and

dissociation (Lawrence and Springer, 1991), ii) high tensile strength (Alon et al., 1995), and iii) capacity of forming adhesion while cell membranes are maintained at relatively high distance by repulsive surface molecules (Patel et al., 1995). Clearly, it is of interest to test this hypothesis by measuring aforementioned parameters (Kaplanski et al., 1993 ; Alon et al., 1995).

Migration on a receptor-bearing surface is another important cellular process : although different cell populations may display varying locomotory behavior, displacement often involves the forward emission of protrusions such as lamellipodia, with subsequent adhesion allowing the cell to contract and detach its rear part from the substratum (Stossel, 1993). Understanding these phenomena at the molecular level requires accurate knowledge of the behavior of adhesion molecules : efficient anchoring of the anterior protrusion requires that a sufficient

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number of bonds be formed to stand tensile forces of several hundreds of piconewtons (Oliver et al., 1994). Detachment of the posterior part is also highly dependent on the mechanical properties of adhesion molecules (Crowley and Horwitz, 1995).

Many adhesion receptors such as selectins (Ushiyama et al., 1993), integrins (Lollo et al., 1993) or members of the immunoglobulin superfamily (van der Merwe et al., 1993) were produced in soluble form and assayed for estimation of the kinetic or equilibrium constants of association with their ligands. However, while these parameters usually provided a satisfactory description of the interaction between soluble molecules, or between a soluble molecular species and surface-bound receptors, it was rapidly clear that more sophisticated tools were required to account for the association between surface-bound molecules. Indeed, as emphasized by Pierres et al. (1996a), the rate of bond formation between membrane-bound molecules is a function of the distance  $d$  between the anchoring points of these molecules. Further, the rate of bond dissociation between surface-bound receptors is usually dependent on the distractive force  $F$  applied on these molecules. Also, the experimental rate of separation of surfaces bound by a ligand-receptor couple is dependent on both the applied force and the motion of surfaces following bond rupture.

Until recently, no experimental approach allowed a direct determination of aforementioned functions. Indeed, standard techniques such as equilibrium dialysis (e.g. Kabat, 1968) or more refined methods such as plasmon resonance based technology (van der Merwe et al., 1993) could not be applied to bound molecules. Also, although some theoretical models of cell adhesion provided a link between association rates and measurable aspects of cell behavior such as contact areas (Bell et al., 1984), adhesion efficiency (Hammer and Lauffenburger, 1987) or binding strength (Dembo et al., 1988), they relied on too many unknown parameters or untested assumptions to allow a safe derivation of molecular properties. This situation emphasizes the remarkable interest of simple theoretical models elaborated by Bell (1978) who provided a fairly simple link between the behavior of soluble and bound adhesion molecules, and suggested some testable

predictions concerning the mechanical strength of molecular bonds. Thus, he estimated at a few tens of piconewtons the force required to rapidly break a single interaction comparable to an antigen-antibody bond.

Remarkably, within a few years, a variety of experimental approaches allowed fairly direct test of Bell's model. Indeed, experiments based on hydrodynamic forces (Tha et al., 1986), soft vesicles used as tunable transducers (Evans et al., 1991), or atomic force microscopy (Florin et al., 1994) demonstrated that the strength of many ligand-receptor interactions was of the order of several tens of piconewtons (or slightly higher values when the high affinity interaction between avidin and biotin was studied). A common feature of these techniques was to study a few or even individual bonds, which alleviated many difficulties hampering the interpretation of previous experiments. Other authors reported on the distance dependence of the energy of interaction between receptors and ligands (Helm et al., 1991), the natural lifetime of weakly stressed bonds (Kaplanski et al., 1993), the precise dependence of the bond lifetime on applied force (Alon et al., 1995) or the distance dependence of association rate (Pierres et al., 1997).

The aim of the present review is therefore twofold : first, we shall discuss the significance of the physical parameters we need define to achieve a satisfactory description of bonding behavior. Second, we shall review recent methodological advances allowing direct measurement of these parameters. For the sake of clarity, we shall sequentially discuss the processes of bond formation and dissociation.

### RATE OF BOND FORMATION

We shall start by following the simple approach used by George Bell (1978). In a solution, the formation of a bond between two molecules A and B is conceptually separated into sequential steps (Figure 1) :



The first step is the formation of a so-called encounter complex between A and B : these molecules are brought into binding distance through mere diffusion. This distance

will be denoted as  $R_{AB}$ . It is expected to be close to the sum of the radii of molecules A and B if these are modeled as spheres.

The second step is bond formation. This is much more complicated, since it requires suitable rotation in order that reactive sites come in close contact ; then the energy barrier separating free and bound states must be overcome by thermal fluctuations.

Although the rate of formation of the bound species C can be calculated analytically with respect to the parameters defined in (1), the exact formula is somewhat awkward. Thus, Bell took advantage of a widely used procedure consisting of considering a steady state phase, where the concentrations of molecules A and B as well as the intermediate complex AB may be considered as fairly constant. He obtained :

$$d[C]/dt = k_+ [A] [B] \quad (2)$$

$$k_+ = d_+ r_+ / (d_+ + r_+) \quad (3)$$

(where the square brackets represent the concentration of any molecular species). There remains to understand the difference between reactions involving free and surface-bound molecules. Bell suggested that the second step might proceed with similar rate between free and bound molecules. Simple estimates based on diffusion equations might thus allow a crude derivation of the rate of association between membrane molecules from the properties of soluble species. This simple view played a major role as a starting point in understanding molecular interactions at the cell surface. However, as will be now discussed, some points must be clarified in order to discuss recent experimental data within the framework of Bell's theory. We shall discuss sequentially the two steps described in equation (1).

### Diffusion phase.

First, let us look at an intuitive representation of the steady state phase : the basic assumption is that the average concentration of species B at distance  $R_{AB}$  from molecules A is some constant  $\kappa [B]$ . If the reaction rate  $r_+$  is very high,  $\kappa$  is expected to be close to zero, and the rate limiting step is the arrival of molecules B in contact with A. The overall reaction is said to be diffusion-

limited. On the contrary, if  $r_+$  is much lower than  $d_+$ , constant  $\kappa$  is close to 1. The steady-state reaction rate can be calculated with a theory first elaborated by Smoluchowski (1917). As briefly described in Appendix I, in a three-dimensional medium :

$$d_+ = 4 \pi (D_A + D_B) (1 - \kappa) R_{AB} \quad (4)$$

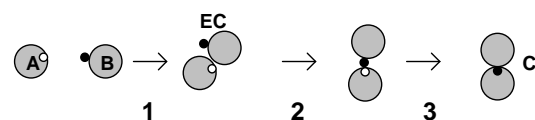
where  $D_A$  and  $D_B$  are the diffusion constants of molecules A and B respectively. Now, if molecules are embedded in a membrane, the surface rate of formation of the encounter complex may be calculated in a similar way, yielding (Appendix I) :

$$d_{+m} = 2\pi (D_{Am} + D_{Bm})(1 - \kappa) / \ln(R_{cell}/R_{AB}) \quad (5)$$

where  $R_{cell}$  is the cell radius. Note that this formula may seem at variance with Bell's equation. However, the logarithm is not very different from unity and there is no order-of-magnitude discrepancy between this and Bell's equation.

### Reaction step.

The reaction step is conceptually much more complicated than the diffusion phase. It seems reasonable to split this process into a rotation step that is required to bring binding sites in contact, and the intermolecular association that is driven by intermolecular potential (Figure 1).



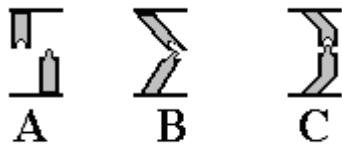
**Figure 1. Association between soluble molecules.** The formation of a complex between molecules A and B may be divided into i) a diffusive step (1) leading to the formation of an encounter complex (EC) and ii) a reaction step, involving molecular reorientation (2) and association of complementary sites (3).

**Molecular rotation.** As pointed out by Bell (1978), the rate constant of some antibody-hapten reactions may be fairly close to the diffusion limit. This may seem somewhat surprising, since it might be thought that only a low proportion of molecular encounters should happen with an orientation compatible with binding (Figure 1). It is therefore of interest to

note that there is some evidence supporting the concept that hydrodynamic (Brune and Kim, 1994) or electrodynamic (Helm et al., 1992) forces between "well designed" molecules might induce couples generating rotation conducive to the acquisition of correct orientation during intermolecular approach.

The problem is to know what happens when molecules are bound to a surface. The following two predictions may be safely suggested :

- If molecules are rigid, only a minimal fraction of orientations may be compatible with binding (Figure 2), resulting in a dramatic decrease of the binding rate as compared to free structures.



**Figure 2. Dependence of bond formation on molecular shape and flexibility.** The association between two surface-bound molecules cannot occur if binding sites are not in contact (A) or if molecular rigidity prevents the acquisition of a suitable orientation of binding sites (B). Therefore, the binding efficiency is expected to be much higher when interacting molecules are sufficiently flexible (C).

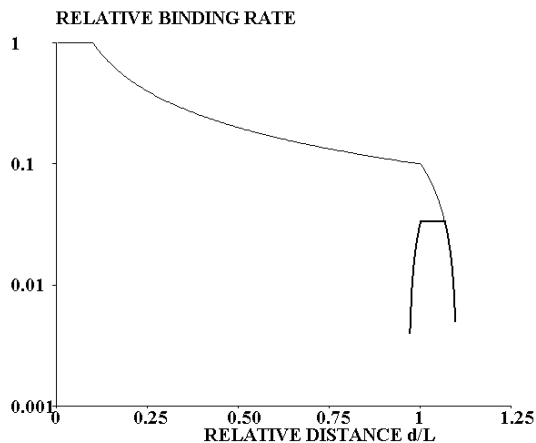
- If adhesion molecules are flexible, the diffusion of binding sites should not be substantially decreased on a membrane. Indeed, if we model these sites as points located on the surface of a rigid spherical molecules of radius  $a$ , the diffusion component due to translation is expected to be equal to  $kT/6\pi\mu a$ , where  $k$  is Boltzmann's constant,  $T$  is the absolute temperature and  $\mu$  is the medium viscosity, whereas the diffusion component due to rotation (i.e. sphere radius times rotational diffusion coefficient) should be  $kT/8\pi\mu a$  (see e. g. Hill, 1960).

The qualitative conclusion suggested by these remarks is that rigid molecules are not expected to interact if they are bound to surfaces. If they are flexible, binding sites are expected to move with comparable velocity in solution and on a membrane. Therefore, the kinetics of bond formation should be proportional to the fraction of available

molecular conformations corresponding to a molecular orientation compatible with binding. As shown on Figure 2, this probability is expected to depend on the distance between the anchoring points of considered molecules.

**Molecular association.** A detailed discussion of the general mechanisms of the kinetics of molecular interactions would not fall into the scope of this review. However, molecular flexibility (as well as the flexibility of anchoring to the membranes) is likely to play a dominant role at this stage. Indeed, according to Eyring's celebrated theory of reaction rates (Eyring, 1935 ; Hill, 1960), the absolute rate of molecular association should be proportional to the rate of passage through an "activation state". Both the probability of formation of this state and the transition rate per activated complex between molecules of comparable size should be inversely proportional to a power of at least 1/2 of the molecular mass. Thus, molecules of about 5 nm radius **rigidly** bound to cells of 5  $\mu\text{m}$  radius should display at least  $10^9$  fold (i.e.  $[5\mu\text{m}/5\text{nm}]^3$ ) lower chemical reaction rates than freely moving molecules. On the contrary, if molecules are **flexible** enough, reactive sites might behave as molecules with an effective mass comparable to the masses of **free** receptors and ligands.

It is therefore concluded that surface-bound molecules will be able to interact efficiently only if they are fairly flexible. In this case, the diffusive rate of binding sites is comparable to that of free molecules, but this diffusion is constrained within a very small volume. The binding rate should therefore be exquisitely sensitive to the surface distance, with a characteristic range comparable to molecular size. These conclusions were, in fact, intuitively obvious, but they must be borne in mind in order to interpret results obtained with the methods that will be described in the second part of this review. A semi-quantitative model for the distance dependence of bond association is presented in appendix II. Theoretical curves for the distance-dependence of association rate between surface-bound molecules are presented on Figure 3.



**Figure 3. Expected distance-dependence of the association rate of surface-bound adhesion molecules.** The crude model described in appendix II was used to estimate the distance dependence of the association rate between a surface coated with freely rotating rod-like molecules and i) a surface coated with rigidly bound ligands with parallel orientation, located in a thin layer (**thick line**), and ii) a surface coated with freely rotating ligands (**thin line**).

### BOND DISSOCIATION.

As emphasized by Bell (1978), the spontaneous rupture of a molecular bond requires that i) a sufficient amount of thermal energy be accumulated to reach the transition state and ii) unbound molecules get separated by diffusion.

When at least one of interacting molecular species is in soluble form, it is reasonable to consider that the debonding process is correctly described by a single numerical constant, that is the off-rate ( $k_-$ ), or probability of rupture per unit of time. When both ligands and receptors are bound to surfaces, two important differences must be considered :

- First, the bond may be subjected to some distractive force  $F$ . The off-rate  $k_-$  must therefore be considered as a function of  $F$  (i.e.  $k_-(F)$ ). Bell (1978) suggested to apply to individual bonds an empirical formula that was obtained by studying the tensile strength of macroscopic bodies (Zurkhov, 1956) :

$$k_-(F) = k_-(0) \exp(\gamma F/kT) \quad (6)$$

Bell argued that the constant  $\gamma$  must represent the molecular range of interaction, and he suggested a tentative value of a few tens of nanometers for the antigen-antibody bond.

Some theoretical justification for equation (6) may be found within the framework of Eyring's theory (see appendix III and Evans and Ritchie, 1997). As shown below, the conclusion that the tensile strength of molecular bonds should be of order of several tens of piconewtons was fully supported by further experimental studies. More recently Evans et al. (1991) suggested the following empirical formula :

$$k_-(F) = v_0 (F/F_0)^a \quad (7)$$

where  $a$  is an empirical parameter describing the mechanical behavior of the considered bond. The important conclusion is that the off-rate is dependent on applied force, leaving as a challenge to experimentalists an accurate determination of this dependence in several representative models.

- Second, free and bound molecules are expected to display quite different behavior. The problem is to know whether rebinding is likely to occur after bond rupture. Indeed, even if a surface-bound molecule is flexible enough to remove its binding site from a receptor with a time scale comparable to that found with a free molecule, the local concentration of the dissociated binding site will remain quite high (the concentration of a molecule constrained in a volume of 10 nm size is of order of 1.7 mM). Thus, the apparent lifetime of a surface-bound molecule subjected to a tensile strength  $F$  may be dependent on the dynamic properties of the particle, i.e. on its effective mass. Indeed, this mass determines the velocity with which the molecule will go away after detachment. This problem may be important if the reciprocal value of the on-rate (i.e. the binding time) is smaller than the time required for the detached surface to move by a molecular length when it is subjected to a distractive force  $F$ .

### EXPERIMENTAL STUDY OF THE INTERACTION BETWEEN SURFACE-BOUND MOLECULES.

We shall now review some recent experimental studies on the interactions between individual surface bound molecules. The basic methodologies that yielded most published information during the last years were reviewed in a recent book (Bongrand, Claesson & Curtis, 1994)

### The surface forces apparatus.

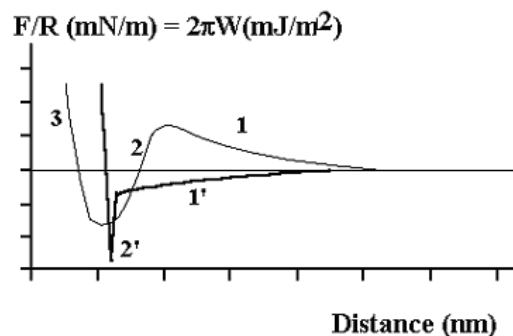
The surface forces apparatus (Israelachvili and Tabor, 1972 ; Israelachvili and Adams, 1978) has been used for more than twenty years to study short-distance interactions between surfaces. The basic principle was to bring into close distance two crossed cylinders coated with silvered mica surfaces, with simultaneous measurement of intersurface distance and interaction force. Three points may be emphasized (Israelachvili, 1991):

- The distance between surfaces is measured by means of an interferometric technique allowing better than angström accuracy, and precise determination of surface deformation (Helm et al., 1989). Note that the interest of using mica is due to the smoothness of this material at the atomic scale.
- The force is measured with an accuracy of order of 10 nanonewtons : this is achieved by combining accurate distance measurement, mounting of a cylinder on a soft spring (cantilever), and control of additional displacement with a piezoelectric device.
- When the radius of curvature  $R$  of the cylinders ( $\sim 1\text{cm}$ ) is much higher than the range of the interaction, it may be shown that the measured force  $F$  is related to the interaction energy  $W$  per unit area through the simple formula :

$$W = F/2\pi R \quad (8)$$

(This is the so-called Derjaguin approximation). The surface forces apparatus (SFA) allowed direct measurement of van der Waals forces in water (Israelachvili and Adams, 1978). It was then applied to surfaces coated with lipid films or macromolecules (see e. g. Luckham and Klein, 1985 ; Claesson, 1994) and yielded very sensitive energy/distance curves. Recently, (Helm et al., 1991 ; Israelachvili et al., 1994), this apparatus was applied to specific interactions between biological molecules. The first model studied was the avidin-biotin association : this was chosen in view of its extremely high affinity. In order to obtain very regular arrays of avidin and biotin binding sites, the authors incorporated these molecules in lipid layers that were deposited on mica surfaces. Other models such as antigen-antibody bonds (Leckband et al., 1995) or nucleic acid

interactions (Pincet et al., 1994) were also studied with this technique. The major conclusion was that specific interactions resulted in very strong attraction within a very narrow range of distances (Figure 4 : the peak width was of order of one angström). The peak value of this attraction could **in principle** allow an absolute determination of the interaction energy, **provided** the surface density of binding sites was determined. The estimate yielded by equation (8) for the avidin-biotin interaction energy is about 17 kT (Helm et al., 1991 ;  $k$  is Boltzmann's constant and  $T$  is the absolute temperature). The binding constant for the avidin-biotin association in solution is about  $10^{15} \text{ M}^{-1}$ . In a study made on the interaction between fluorescyl groups and



**Figure 4. Study of molecular interactions with the surface forces apparatus.** The measured force  $F$  is proportional to the interaction energy  $W$  per unit area. The thin line represents a typical interaction curve between two charged surfaces. When these surfaces are approached, electrostatic repulsion (1) is overcome by van der Waals attraction (2), followed by short range repulsion (see e. g. Marra and Israelachvili, 1985). Typical ligand receptor interactions (thick line) sometimes displayed long-range "steering" attraction (Leckband et al., 1992 & 1995) (1') followed by a very sharp attraction (2'). Note that the force and distance scales are arbitrary.

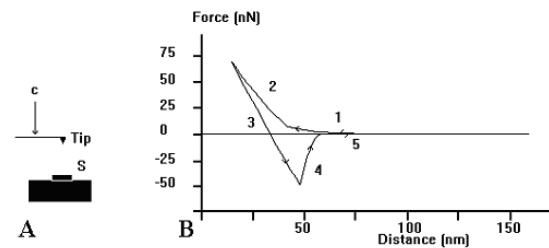
a specific antibody (with an affinity of  $5 \times 10^9 \text{ M}^{-1}$ ), assuming a surface area of  $45 \text{ nm}^2$  per antibody site, with 75 % of active sites, the binding energy measured with the surface forces apparatus is about 6 kT (Leckband et al., 1995). Note that the relationship between measured binding energy and affinity constant is not at all straightforward. This point is discussed in appendix IV.

Another important finding (Leckband et al., 1992 & 1995) was that a weak attraction

between protein sites was detectable when they were separated by a distance as high as 8.5 nm (Leckband et al., 1992). Thus, as emphasized by the authors, when two adhesion molecules approach each other through diffusion, electrodynamic steering forces may ensure suitable orientation in order that binding sites are facing each other when molecules come in contact. Interestingly, these forces are due to a particular spatial organisation of the molecule rather than the existence of average opposite charges on ligand and receptor molecules, since this force disappeared on protein denaturation (Leckband et al., 1995).

Another interesting finding was that the specific interaction between avidin and biotin molecules could be detected only when they were deposited on fluid, not solid, monolayers, thus emphasizing the importance of a molecular match in short range interactions (see also McGuiggan and Israelachvili, 1990). The importance of molecular length and flexibility is indeed obvious in many biological models.

A final advantage of the surface forces apparatus is that the accurate control of surface distance may yield very safe information on molecular conformation on interfaces. However, there are some basic limitations with this technique : first, since many molecular interactions are averaged, it has not been possible at the present time to derive kinetic parameters of bond formation and dissociation. Second, avidin-biotin interaction was found to be stronger than the cohesive strength of molecular layers, thus hampering precise study of the detachment process. Third, quantitative discrimination between nonspecific van der Waals forces and specific effects may be somewhat complicated. Fourth, it seems difficult to apply this methodology to irregular surfaces such as are found in biological systems. It is therefore interesting to compare the data obtained with this approach to results provided by other techniques. During the last few years, several authors obtained very accurate information on individual ligand-receptor bonds with atomic force microscopy.



**Figure 5. Study of molecular interactions with atomic force microscopy.** **A** : The tip of nanometer width is glued to a soft cantilever (c) whose deflection is continuously monitored by a suitable sensor during approach to the sample S. It is thus possible to obtain force/distance curves (**B**). If the sample and tip are coated with specific ligands and receptors, the repulsion that is observed during approach (steps (1) and (2)) is replaced by attraction (3) when the distance is increased. The bond rupture (4) appears as a single (figure) or multiple (not shown) jump whose amplitude represents the bond strength.

#### Atomic force microscopy.

As briefly sketched on Fig. 5, the basic principle of an atomic force microscope (Binnig et al., 1982) is deceptively simple (see e. g. Erlandsson & Olsson, 1994, for a brief description). A very sharp needle (the tip width may be on the nanometer scale) is mounted on a soft cantilever (typically of order of 0.1 N/m stiffness) whose position may be detected with angstrom accuracy. The tip is moved near a suitable material sample. A piezoelectric device is used to control the tip-to-sample distance. Now, the usual way of using an atomic force microscope (AFM) is to scan the sample by lateral displacement of the tip while the interaction force is maintained constant by a feedback mechanism. The actual tip-to-surface distance is easily calculated since imposed displacement and cantilever deformation are known. It is thus possible to obtain an **image** with nanometer resolution. However, the AFM was recently applied to the study of intermolecular forces by derivatizing the tip and the surface with complementary ligand and receptor sites, and varying only the distance between these structures. It was thus possible to derive force/distance curves. The rupture of specific bonds resulted in sharp jumps of the tip (Figure 5), whose amplitude might be translated in a force that was defined as the bond mechanical strength.

In contrast with the surface forces apparatus, the AFM does not allow an

**absolute** determination of the distance between surfaces. However, there are several advantages with this technique : first, there is no need for perfectly smooth surfaces, and it might well be possible to study bond formation on the surface of actual cells (Hinterdorfer et al., 1996). Second, since the interaction area is much lower than with the SFA, the relative importance of van der Waals forces is much less, and no complicated calculation is required to subtract the effect of these interactions. Third, hydrodynamic interactions are also much less important, making it possible to impose fairly rapid relative displacement of the tip and the sample. As a consequence, this technique might in principle yield kinetic information on individual bonds. Indeed, in a typical experiment, the AFM tip velocity may be of order of 100 nm/s, which is fiftyfold higher than was achieved with the SFA (Leckband et al., 1995).

Further, while the theoretical force and displacement sensitivity of the AFM might be of order of 0.01 pN and 0.01 nm respectively (Lee et al., 1994), several authors measured adhesion forces with a sensitivity of a few piconewtons. Indeed, Hoh et al. (1992) may have detected the rupture of individual hydrogen bonds in aqueous medium. Recently, much interesting information was reported on ligand-receptor interactions :

The streptavidin-biotin bond was studied by several authors (Florin et al., 1994 ; Lee et al., 1994, Moy et al., 1994). When interacting surfaces were coated with limited densities of avidin and biotin groups, the rupture of specific bonds appeared as sharp jumps of the tip. Two arguments were used to support the view that **individual** bonds were indeed detected. First, it was argued that when a limited proportion of approaches between the tip and sample resulted in binding, the bond number must be low (Lee et al., 1994). Second, when histograms of the distribution of rupture forces were drawn, there appeared a series of quantized peaks that were multiples of a single value (Florin et al., 1994). The reported value of the rupture strength of the avidin-biotin bond varied between 160 pN (Florin et al., 1994), 257 pN (Moy et al., 1994) and 300-400 pN (Lee et al., 1994). The force required to separate paired adenine and thymine groups was estimated at 54 pN (Boland and Ratner, 1995). Other authors estimated at 400 pN the rupture force of association between two cell

adhesion proteoglycans (Dammer et al., 1995). The rupture force of interaction between biotin and polyclonal anti-biotin antibodies was estimated at 111.5 pN (Dammer et al., 1996), and the rupture force of the interaction between human albumin and specific antibodies was about 240 pN (Hinterdorfer et al., 1996). Interestingly, the latter authors reported that this force of 240 pN reduced the bond lifetime by a factor of  $8 \times 10^5$  as compared to the value measured on soluble molecules. Here are some points of practical or theoretical interest :

First, it was noted that the measured bond strength was dependent on the cantilever stiffness (Lee et al., 1994). This point was indeed consistent with a previous report by Evans et al., (1991) who emphasized that bond rupture was a stochastic event whose frequency was dependent on applied force. Further, Evans et al. (1994) suggested an empirical relationship between the level of force "f" for most frequent rupture depends and the rate of loading. It is therefore not surprising that when a very low loading rate was used to probe the streptavidin-biotin interaction with so-called biomembrane-force probe (BFP : Evans and Ritchie, 1997), the detachment force was about 50 pN (Merkel et al., 1995).

Second, it was reported that, in contrast with studies made with the SFA on lipid-incorporated adhesion molecules, adhesion receptors were not damaged with AFM studies, which allowed to perform hundreds or thousands of attachment/detachment cycles without any need for a lateral displacement of the tip (Florin et al., 1994 ; Hinterdorfer et al., 1996).

Third, the importance of using adhesion molecules with sufficient flexibility (Hinterdorfer et al., 1996) or a soft substratum (Florin et al., 1994) was emphasized, thus supporting the concept that geometrical constraints may play an essential role in the efficiency of interaction between surface-bound molecules.

Fourth, the significance of measured forces was studied by comparing the binding strength of interaction between avidin and a series of biotin derivatives : a linear relationship was found between the force and the dissociation enthalpy, not the free energy (Moy et al., 1994 ; Chilkoti et al., 1995). This



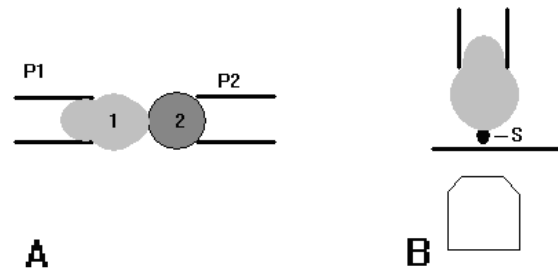
yielded an estimate of 0.95 nm for the rupture length (Moy et al., 1994). Also, these results suggested that detachment acted as an adiabatic process (i.e. this process did not involve any energy exchange between the ligand-receptor couple and the solvent). This point was by no means obvious, and it had to be clarified in order to allow significant comparison between experimental data and results of computer simulation (Grubmüller et al., 1996).

Fifth, whereas initial studies were focussed on bond rupture, Hinterdorfer et al. (1996) recently attempted to estimate the rate of bond formation between an AFM tip coated with a few antibody molecules and antigen-coated surfaces. Thus, they obtained an estimate of  $5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  for the association rate, which fell within the range of antigen-antibody association constants.

#### Use of lipid vesicles as transducers.

The use of lipid vesicles as transducers was pioneered by Evans et al. (1991). They used micropipettes to approach red blood cells coated with limiting densities of various adhesion molecules. One of these cells was stiffened by chemical cross-linking or aspiration with high pressure. The other one was maintained with moderate pressure. After a contact of several tens of seconds, a pipette was pulled, resulting in the application of an increasing disruptive force. This force could be calculated by analysis of the cell deformation (Figure 6). The rupture time, and the corresponding force were then recorded. An important finding was that the force required for rapid (i. e. a few second) bond rupture was similar when adhesion was mediated by different antigen-antibody couples or lectin-sugar interactions. This rupture force was close to 10-20 pN.

The authors later improved this method by biochemically glueing a microscopic bead to the surface of a lipid vesicle : accurate determination of the bead position could thus be achieved with interference reflection microscopy (Figure 6). Further, the bead was approached to a test surface that could be moved with a piezo-electric device.



**Figure 6. Use of soft vesicles as tunable transducers to monitor molecular interactions.**

(A) The soft vesicle (1) is manoeuvred into contact with a rigid sphere (2) with two micropipettes (P<sub>1</sub> and P<sub>2</sub>). After bond formation, the distance between pipettes is increased under continuous video recording. The force exerted on bond rupture is derived by analyzing the deformation of the soft vesicle (Evans et al., 1991). (B) a small rigid sphere S is chemically glued to a soft vesicle. The distance between the sphere and the surface is measured with interference reflection microscopy (Evans et al., 1994).

The interest of this technology is i) to take advantage of a tunable transducer with a wide range of forces and ii) to allow some visual control of surface position.

#### Use of hydrodynamic flow to study bond formation and dissociation.

The first experimental check of Bell's theory was provided by Tha et al. (1986). They made use of a device called the "traveling microtube" that had been developed by H. Goldsmith for some years. The basic principle was to drive doublets of antibody-agglutinated erythrocytes into a glass capillary tube with **very low** pressure. A microscope was used to monitor the motion of individual doublets. The trick was to settle the tube on a moving stage with a velocity opposite to the flow. The observed doublet was thus immobile with respect to the microscope and could be monitored during a prolonged period of time. Also, erythrocytes were made spherical by incubation in hypotonic media, which allowed precise calculation of forces with theoretical results from fluid mechanics. Recording the motion gave direct information on the hydrodynamic distractive force and doublet lifetime. Another important point was to use limiting concentrations of agglutinating antibodies (in order that cells be held together by a few and even a single bond). It was thus possible to observe doublet rupture in presence of weak hydrodynamic forces. The strength of

an antigen-antibody bond was estimated at a few tens of piconewtons. These results were essentially confirmed in a later study based on improved methodology allowing rapid variation of the applied force, with a cone-and-plate rheoscope (Tees et al., 1993 ; Goldsmith et al., 1994), and replacement of polyclonal with monoclonal antibodies. Finally, since there remained the possibility that cell separation might involve the uprooting of membrane bound antigens rather than rupture of the antigen-antibody bond (Bell, 1978 ; Evans et al., 1991), experiments were resumed in the same laboratory with ligand-derivatized latex spheres. The motion of flowing doublets was followed with a range of shear rates. Computer simulation was performed to fit experimental values of bond lifetime with predictions from Bell's theory. The natural lifetime (i.e. at zero force) of the interaction between a polysaccharide antigen and antibody bonds was found to be 25 seconds, with an empirical interaction range  $\gamma$  (equation 6) of 0.12 nm (Tees and Goldsmith, 1996). The interaction between immunoglobulin G and protein G, a natural IgG receptor of bacterial origin, was also studied : the natural lifetime of the interaction was 175 seconds and the interaction range  $\gamma$  was 0.39 nm (Kwong et al., 1996).

A major limitation of the above experiments is that it was difficult to obtain accurate information on short-lived bonds. This difficulty could be overcome with a parallel-plate flow chamber : the basic idea was to observe the motion of receptor-coated cells or particles along ligand-coated surfaces with a very low hydrodynamic force (say a few piconewtons, i.e. less than the rupture strength of a single bond). It was thus reasoned that single molecular bonds should result in the formation of detectable arrests. When blood polymorphonuclear neutrophils were driven along activated endothelial cells (Kaplanski et al., 1993), they displayed transient arrests with an average lifetime of about 2 seconds, and these binding events were inhibited when endothelial cells were treated with anti-E-selectin antibodies. More extensive data were obtained on a similar system by Alon et al. (1995) who studied the binding of neutrophils with surfaces coated with various densities of P-selectin, with different values of the flow rate : they estimated at 1 second the natural

lifetime of P-selectin-ligand bond, and the bond interaction distance (i.e. Bell's parameter  $\gamma$ ) was 0.05 nm.

Since an accurate study of cell motion was made difficult by velocity variations due to cell surface asperities, Pierres et al. (1994 & 1995) used the same technique to study the displacement of **spherical** particles coated with anti-rabbit immunoglobulins along surfaces derivatized with rabbit Ig. Unexpectedly, they observed short-term arrests (with a lifetime ranging between about one and two seconds), whereas the attachment of soluble antibodies lasted several hours. A quantitative analysis of arrest duration strongly suggested the existence of a biphasic binding process, with formation of an intermediate adhesion state. When the shear rate was increased, the lifetime of the intermediate state decreased with an empirical interaction range  $\gamma$  of about 0.08 nm (unpublished data). It must be emphasized that this finding was in line with previously reported data (Beeson and McConnell, 1994). Note that it is not surprising that these intermediate states were revealed with the flow chamber technology rather than the AFM : indeed, when a sphere moves within binding distance of a surface with a velocity of 10  $\mu\text{m/s}$ , the relative velocity between the receptors and ligands is of order of 5  $\mu\text{m/s}$  (Goldman et al., 1967), as compared to a fiftyfold lower value of the AFM tip velocity.

In a later study, Pierres et al. (1996b) improved the performance of the flow chamber methodology by using image analysis to estimate bead position with 0.1  $\mu\text{m}$  accuracy and a time resolution of 5 milliseconds. It was thus possible to study short term interactions between recombinant CD2 molecules and their ligand CD48. These molecules are expected to play a role in the initial interaction between T lymphocytes and antigen-presenting cells. The natural bond lifetime was of the order of one tenth of a second with a bond range parameter  $\gamma$  of 0.13 nm (Pierres et al., 1996c).

Recently, the flow chamber was used to study the on-rate of bond formation (Pierres et al., 1997) : CD48-coated spheres were driven along CD2-derivatized surfaces. About 2,500 individual trajectories were recorded, yielding 350,000 positions. Results were used to derive a relationship between instantaneous sphere velocity and binding probability. Numerical data provided by Goldman et al.

(1967) were then used to plot the binding probability versus the sphere-to-surface distance. The binding site density was then determined, which allowed an absolute estimate of the variations of the rate of bond formation between CD2 and CD48 molecules versus the distance between the anchoring points of these molecules. The association rate was  $0.03 \text{ second}^{-1}$  at 10 nm separation, and it was inversely proportional to the cube of the distance. This finding is not inconsistent with the crude model described in Appendix II.

In conclusion, the flow chamber allowed quantitative determination of both bond rupture rate as a function of applied force, and bond formation kinetics as a function of distance. Also, the occurrence of intermediate binding states was readily demonstrated.

#### Other methods.

Although they were less extensively used than aforementioned approaches, some techniques were demonstrated to yield valuable information on the association between bound adhesion molecules.

A rapidly developing methodology consists of exerting a force of several piconewtons on a microscopic object by focussing a laser beam on this object on the stage of a microscope (these devices are called laser traps or "optical tweezers"). The force is generated by the deflection of photons when light rays are diffracted (see e. g. Ashkin, 1992). This technique was recently used by Miyata et al. (1996) to study the strength and lifetime of actin- $\alpha$ -actinin interaction. The authors reported very heterogeneous bond lifetime, with a mean duration of 0.52 second. The force dependence was complex, with wide variations of the dissociation constant, which made very difficult a quantitative check of Bell's model. The binding range  $\gamma$  was estimated between 0.05 nm and 0.3 nm.

A recently described method consisted of observing the thermal motion of immunoglobulin-coated spheres near protein A-coated surfaces (protein A is a natural immunoglobulin receptor of microbial origin). Total internal reflection microscopy allowed to monitor elevations as small as 1 nm. The authors took advantage of Boltzmann's distribution to derive an energy/distance

relationship between beads and the surface (Liebert and Prieve, 1995).

#### CONCLUSION.

Experimental studies made on cell adhesion have long suggested that a single parameter such as the affinity constant could not give a complete account of the behavior of cell surface receptors. Recent methodological advances recently yielded an impressive amount of information on ligand receptor interactions, with fairly direct determination of the relationship between i) dissociation rate and applied force, and ii) association rate and distance of interacting molecules. It may thus be expected that accurate comparison between experimental data and theoretical predictions, including results from computer simulation experiments, will dramatically improve our understanding of the mechanisms of macromolecule interactions within the next few years. This will allow in-depth understanding of the relationship between the structure and function of the multiple adhesion molecules that have recently been characterized.

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## APPENDIX I

### Steady state kinetics of formation of an encounter complex.

Following the approach initiated by Smoluchowski (1917), we consider the mean concentration  $c_B(r,t)$  of molecules B at time t and at distance r from the center of gravity of a molecule A. The diffusion equation may be written as :

$$\vec{J} = -(D_A + D_B) \vec{grad} c_B(r,t) \quad (\text{AI-1})$$

where  $\vec{J}$  is the mean particle flux due to diffusion. Note that  $(D_A + D_B)$  may be viewed as the "mutual" diffusion constant of A and B. Under stationary conditions,  $c_B(r,t)$  is independent of time, and the conservation equations reads :

$$\text{div} \vec{J} = 0 \quad (\text{AI-2})$$

using polar coordinates and taking advantage of the simple spherical symmetry of the system, we obtain

$$\frac{d^2}{dr^2}(rc_B) = 0 \quad (\text{AI-3})$$

thus,  $rc_B$  must be a first order polynoma of r. Using the simple boundary conditions  $c_B = c_{B0}$  at infinity and  $c_B = \kappa c_{B0}$  when r is equal to  $R_{AB}$ , we obtain :

$$c_B(r) = c_{B0} (1 - [1-\kappa]R_{AB}/r) \quad (\text{AI-4})$$

Equation (AI-1) may then be used to calculate the number of type-B molecules entering the sphere of center A and radius  $R_{AB}$  per unit of time :

$$4\pi R_{AB}^2 J = 4\pi c_{B0} (1-\kappa) R_{AB} \quad (\text{AI-5})$$

Since the rate of formation of encounter complexes is simply the product of the above quantity times the concentration of type-A molecules, Equation (AI-5) readily yields Equation (4). Note that the derivation of the time-dependent form of  $c(r,t)$  is described in Appendix 2 of the review by Bongrand et al. (1982).

If the same mode of reasoning is used under 2-dimensional conditions, Equation (AI-3) is replaced with :

$$\frac{d}{dr} \left( r \frac{dc}{dr} \right) = 0 \quad (\text{AI-6})$$

this equation yields :

$$c(r) = a \ln(r) + b \quad (\text{AI-7})$$

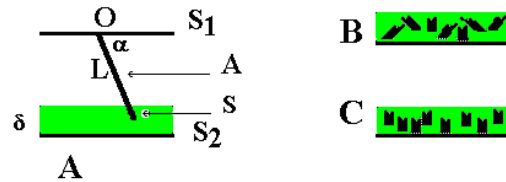
where  $a$  and  $b$  are constants. There is a difficulty since there is no finite limit at infinity unless  $a$  is zero. This means that there is a problem with the steady-state assumption. However, we may write that  $c_B$  is  $c_{B0}$  at some distance  $R$  that is of the order of the cell radius, yielding equation (5). Note that the logarithm is only weakly dependent on the choice of  $R_{AB}$ .

## APPENDIX II

### Expected dependence of the association rate on the distance between cell surfaces.

If we assume that rotation is entirely free, all orientations of molecule A have similar probability. The association rate should thus be proportional to the fraction of orientations that are compatible with binding. This fraction can be estimated with simple geometrical arguments : indeed, when molecule A is rotating, the binding site S remains on a sphere of center O and radius L. The binding probability is thus equal to the ratio between the area of the subset of this sphere consistent with binding and the area of the sphere segment that is accessible to S. This ratio may be easily calculated by noticing that the surface of a segment of a sphere of radius L

The aim of this appendix is to describe a simple model in order to illustrate the potential influence of the shape and flexibility of surface-bound adhesion molecules on binding rate. Consider the initial interaction between molecule A, viewed as a rigid rod of length L freely rotating around its anchor O on surface  $S_1$ , and a surface coated with a high density of receptor sites remaining in a slab parallel to surface  $S_2$  located at distance  $d$  from  $S_1$  (Figure 7). Since the rotation of molecule A is expected to be much much more rapid than lateral diffusion (see e.g. Bell, 1978), this diffusion should have no influence on binding provided the receptor density is high enough that A can encounter receptors as soon as its binding site enters the receptor zone. For the sake of simplicity, we may thus assume that O is fixed on  $S_1$ .



**Figure 7. theoretical model for the association between surface-bound adhesion receptors.** (A) Surface  $S_1$  is coated with rod-like molecules of length L, freely rotating at their anchoring point O. Ligand sites are considered as smeared in a region of thickness  $\delta$  on the surface  $S_2$  located at distance  $d$  from  $S_1$ . Two different cases were considered quantitatively (as plotted on Figure 3), with free orientation of binding sites in  $S_2$  (B) or parallel orientation of these sites (C).

delimited by two planes separated by a distance  $d$  is  $2\pi Ld$ . Two models may be considered :

- First, we may assume that receptors are rigidly bound to surface  $S_2$  with parallel orientation. We assume that binding is possible only if the angle between molecule A and the normal to  $S_2$  is smaller than some limiting angle  $\epsilon$ . As shown on Figure 7, binding will be possible only if distance  $d$  between surfaces is comprised between  $L+\delta$  and  $(L-\delta)\cos\epsilon$ , and the binding rate will display a sharp maximum at distance close to L. A numerical example (corresponding to  $\epsilon=7.5^\circ$  and  $\delta=L/10$ ) is shown on Figure 3.

- Second, we may assume that receptors are freely rotating. Binding will then be possible when S is within the binding zone. A

numerical binding example (corresponding to  $\delta = L/10$ ) is shown on Fig 3.

### APPENDIX III

#### Simple model for the detachment rate of a bound molecules.

The aim of this appendix is to remind the reader of the principles of Eyring's theory of the absolute rate of chemical reactions, since this may be a basis for Bell's hypothesis on the force dependence of reaction rates (see Eyring, 1935 ; Eyring et al., 1944 ; Hill, 1960). The reader is referred to a recent paper by Evans and Ritchie (1997) for a much more complete discussion including hydrodynamic effects and additional information provided by computer simulation.

We shall illustrate the basic ideas by considering the simple problem of escape of a molecule bound to a receptor site on a surface

The first step is to make use of normal coordinate analysis (a standard procedure in mechanics) and deal with a one-dimensional problem. The principle is as follows : the molecule position is described by many coordinates ; for example, if the molecule were a material point, these would be standard  $x$ ,  $y$  and  $z$  coordinates. The position of a rigid body is in general described by six coordinates (i.e. the position of the center of gravity and three angular parameters). The potential energy of a system with  $n$  "coordinates" may be viewed as a surface in a space of  $(n+1)$  dimension. A bound state will appear as a "basin", and the body motion will be represented as a moving point in this  $(n+1)$ -dimensional space. Due to thermal motion, the energy will fluctuate and allow the body to escape from the basin. The most probable trajectory will be through the line allowing minimal energy increase, and this line may be viewed as the bottom of a valley. It is thus advisable to change the coordinates describing the molecule motion and consider the coordinate along the escape trajectory. The critical point is the passage through the local maximum corresponding to a so-called "transition state".

The second step is to use standard results from statistical thermodynamics to estimate the probability that the particle reaches the transition state. Using the one dimensional representation, we view the

system as a particle with position  $x$  and momentum  $p$  equal to  $m \cdot dx/dt$  (where  $m$  is the particle mass). Thus, the particle state may be represented as a small point in a two dimensional space that is called the "phase space". It is known from quantum mechanics that the particle state must be viewed as a small region of area  $h$  (i.e. Plank's constant) rather than a point. The probability of finding the particle in a region with position  $x$  and momentum  $p$  is related to its total energy ( $U(x)+p^2/2m$ ) through the well known Boltzmann's formula :

$$P(x,p) = (1/Z) \exp[-(U(x)+p^2/2m)/kT] \quad dx dp/h \quad (\text{AIII-1})$$

where  $k$  is Boltzmann's constant,  $T$  is the absolute temperature, and  $Z$  is a normalization parameter that is called the partition function. The assumption that this equation is valid relies on the hypothesis that particle escape is a rare event, and the particle can pass through many states before it reaches the transition state.

The third step consists of calculating the mean number of particles passing through the transition state per unit of time ; it is well known that given a homogeneous population of particles of one dimensional density  $c$  (i.e. number of particles per unit length) and velocity  $v$ , the corresponding flux is  $cv$  particles per second. Using equation (AIII-1) and integrating over all possible velocities with statistical weight, we obtain the following flux :

$$J = c(x) \int_0^{\infty} P(x, p)(p/m) dp / \int_{-\infty}^{\infty} P(x, p) dp \quad (\text{AIII-2})$$

The density  $c(x)$  may be estimated with equation (AIII-1) :

$$c(x) = \exp(-U^*/kT) / \int_{-\infty}^{\infty} \exp(-U(x)/kT) dx \quad (\text{AIII-3})$$

Now, if we assume that  $kT$  is much lower than  $U^*$ , the essential contribution to the integral in (AIII-3) comes from locations close to the bottom of the basin. In a first approximation, we may approximate  $U$  as  $(1/2)\lambda x^2$ ,



corresponding to an harmonic potential. After straightforward integration, we obtain :

$$J = (1/2\pi) (\lambda/m)^{1/2} \exp(-U^*/kT) \quad (\text{AIII-4})$$

This formula is equivalent to Eq.11-16 from Hill (1960). Now, if the particle is subjected to a distractive force  $F$ ,  $U(x)$  is replaced with  $U - Fx$ . Assuming that the barrier  $U^* - F\delta$  remains substantially higher than  $kT$ , we obtain :

$$J = (1/2\pi) (\lambda/m)^{1/2} \exp(-F^2/2\lambda kT) \exp(-(U^* - F\delta)/kT) \quad (\text{AIII-5})$$

This is equivalent to Bell's formula, provided  $F/\lambda$  is much lower than the range  $\delta$  of the interaction, which is equivalent to the assumption that  $F\delta$  is much lower than  $U^*$  within the framework of the harmonic approximation.

#### APPENDIX IV

##### Expected difference between the affinity constant of interactions between free and bound molecules.

The basic principles we shall briefly recall were lucidly emphasized by Page and Jencks (1971). Let us consider the reaction of association between two soluble molecules A and B :



The affinity constant  $K_a$  is related to the standard free energy variation with the following formula :

$$K_a = \exp(-\Delta F^\circ/RT) \quad (\text{AIV-2})$$

where  $\Delta F^\circ$  is the free energy variation when one mole of molecule A is combined with one mole of molecule B in a large reservoir where the concentrations of all three molecular species A, B and AB are one molar. This free energy variation  $\Delta F^\circ$  is the sum of the "intrinsic" reaction free energy  $\Delta F^\circ_i$  generated by intermolecular forces during the association between binding sites, and a contribution called  $\Delta F^\circ_m$  originating from the translational and rotational motions associated to the thermal motion.

$$\Delta F^\circ = \Delta F^\circ_i + \Delta F^\circ_m \quad (\text{AIV-3})$$

Now, the latter term can be readily calculated with basic principles from statistical mechanics (see e. g. Hill, 1960). This is dependent on the molecule shape and size. In order to convey a feeling for the order of magnitude of this contribution, we shall only consider the (fairly unrealistic) case of two point-like molecules of mass  $M_A$  and  $M_B$  merging into a material point of mass  $(M_A + M_B)$ . Using the classical Sackur-Tetrode formula, we obtain :

$$\Delta F^\circ_m = RT \ln\{V/h^3 (2\pi kT/M_A)^{3/2}\} - (3/2) RT \ln\{(M_A + M_B)/M_B\} \quad (\text{AIV-4})$$

Where  $h$  is Planck constant,  $V$  is the available volume per mole, i.e. 1 liter under standard conditions,  $k$  is Boltzmann constant,  $T$  is the absolute temperature,  $R$  is the perfect gas constant. Two major conclusions may be drawn for this formula. First, the translational free energy is only weakly dependent on the ratio  $M_A/M_B$ . Second, if two molecules are rigidly bound to macroscopic bodies with a mass higher than theirs by a factor of 1,000,000, the affinity constant might be accordingly increased. However, if molecules are rigid and rigidly bound, the association rate may be quite low.