Interaction of Divalent Antibody with Cell Surface Antigens†

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ABSTRACT: This paper presents a thermodynamic description of the interaction between divalent protein in solution and monovalent ligand attached to a cell membrane, but freely mobile therein. The intrinsic differences between this system and interaction which takes place in a uniform bulk solution are discussed. Specific problems which are addressed include the determination of the number of ligand sites on a specific cell system, comparison of several cell systems in terms of common antigenic components, comparison of different antibody preparations, and attempts to study solubilized cell-surface antigens using established serological methodology.

Studies of the interaction between antibodies and cell surfaces are in widespread use in both immunological and biochemical research. Antibodies are multivalent proteins, and cell-surface antigens—while they may be monovalent—are restricted to a confined volume element and are not of uniform concentrations throughout a bulk solution. These systems can be described rigorously using thermodynamic principles, but the description differs from that of protein–ligand interactions when both species have uniform concentrations in the bulk solution and at least one species is monovalent. This difference is often not fully appreciated despite the fact that any interpretation of equilibrium data rests on an understanding of the thermodynamic principles involved.

In this paper the following specific subjects are dealt with in a rigorous manner and simplifying assumptions are considered in each case: (1) interaction between monovalent protein and monovalent ligand in homogeneous solution; (2) interaction between divalent protein and monovalent ligand in homogeneous solution; (3) interaction between divalent protein in solution and monovalent ligand on the cell surface; (4) attempts to compare relative amounts of a surface antigen common to different cell types; (5) attempts to compare different antibody preparations based on their interaction with a specific cell system; (6) determination of total antigenic sites per cell; (7) comparisons of solubilized antigen with membrane-bound antigen.

The simplest possible system has been chosen to illustrate the thermodynamic principles involved in antibody–cell-surface interactions—that of divalent IgG and a freely mobile membrane-bound antigen which is monovalent. (Extension of the mathematical treatment to antibodies of higher valency follows directly from the discussion.) Even in this case unambiguous interpretation of binding data is often not possible.

Theoretical equations describing the interactions between multivalent antigen in solution and divalent antibody on a cell surface have been published by others (e.g., Bell, 1974; Bell & De Lisi, 1974; De Lisi & Perelson, 1976; Dembo & Goldstein, 1978). They deal with a considerably more complex system than those described in the present paper, and derivations of appropriate mathematical expressions were often based on a kinetic approach rather than a direct thermodynamic analysis. Fundamental principles and exact meaning of experimental parameters are often obscured as the degree of complexity of a model system is increased.

Results

(1) Monovalent Protein and Monovalent Ligand. The interaction between monovalent protein and monovalent ligand is symmetrical, i.e., the mathematical expression for binding of ligand to protein as a function of free-ligand concentration is identical with that for the binding of protein to ligand as a function of free-protein concentration.

We can write the equilibrium expression

$$P + L \rightleftharpoons PL \tag{1}$$

$$K = [PL]/[P][L] \tag{2}$$

where [P] = unreacted protein, [L] = unbound ligand, and [PL] = complex, all in units of moles/liter. By definition

$$[P_T] = [P] + [PL] \tag{3a}$$

and

$$[L_T] = [L] + [PL] \tag{3b}$$

and it follows directly from eq 2 and 3 that

$$\frac{[PL]}{[P_T]} = \frac{K[L]}{1 + K[L]} \tag{4a}$$

or

$$\frac{[PL]}{[P_T]} = \frac{K[P]}{1 + K[P]} \tag{4b}$$

At half-saturation $[P] = [PL] = {}^1/_2[P_T]$ and

$$[L] = 1/K \tag{5}$$

If both ligand and protein are monovalent, there is no difference between association in solution and association when one species is cell bound. This situation is comparable to the binding of small ions or molecules to a protein with multiple, monovalent sites (Steinhardt & Reynolds, 1969; Tanford, 1961; Scatchard, 1949). Because of symmetry one can treat the association between monovalent protein and "s" sites of a cell-surface monovalent ligand in terms of the relation

$$\bar{\lambda} = \frac{\sum_{i=1}^{s} i[CP_i]}{[C_T]} = \frac{sK[P]}{1 + K[P]} \tag{6}$$

where $\bar{\lambda}$ = average number of bound protein molecules/cell, $[CP_i]$ = concentration of complex between the cell and i molecules of protein, $[C_T]$ = total concentration of cells/liter, and [P] = concentration of unreacted protein. The following relationships hold: $s[C_T] = [L_T]$ and $\sum_{i=1}^{s} i[CP_i] = [PL]$ of eq 4. If one has determined $\bar{\lambda}$ as a function of [P], s is obtained by extrapolation to high values of [P] and $K = 1/[P]$ when $\bar{\lambda} = s/2$.

(2) Divalent Protein and Monovalent Ligand in Solution. The sequential reaction of a protein containing two identical binding sites with a monovalent ligand is no longer symme-

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trical. Expressions relating complex formation to free protein [P] are complicated algebraic functions, and simple expressions are obtained only if binding is expressed as a function of [L]:

$$P + L \rightleftharpoons PL_1 \qquad k_1 = [PL_1]/[P][L] \tag{7}$$
$$PL_1 + L \rightleftharpoons PL_2 \qquad k_2 = [PL_2]/[PL_1][L]$$

(k_1 and k_2 are used as the sequential equilibrium constants in order to distinguish them from K_1 and K_2 in the next section).

We can define a binding parameter, $\bar{\nu}$, which represents the total moles of ligand bound/total moles of protein:

$$\bar{\nu} = \frac{[PL_1] + 2[PL_2]}{[P_T]} = \frac{k_1[L] + 2k_1k_2[L]^2}{1 + k_1[L] + k_1k_2[L]^2} \tag{8}$$

This expression can be simplified if the two sites are identical and independent since in this case $k_1 = 2K$ and $k_2 = {}^1/_2K$. K is the association constant which would apply to the same sites present on univalent protein. Equation 8 then reduces to

$$\bar{\nu} = \frac{2K[L]}{1 + K[L]} \tag{9}$$

At half-saturation of the two binding sites, $\bar{\nu} = 1$ and $[L]_{\bar{\nu}=1} = 1/K$. However, if we express the binding in terms of unreacted protein [P], we obtain from eq 7

$$\frac{[P]}{[P_T]} = \frac{1}{1 + 2K[L] + K^2[L]^2} = \frac{1}{(1 + K[L])^2} \tag{10}$$

At $[P] = {}^1/_2[P_T]$, $[L]_{1/2} = (2^{1/2} - 1)/K = 0.414/K$. The ligand concentration at $\bar{\nu} = 1$ where half the binding sites are occupied does not correspond to the ligand concentration at $[P] = {}^1/_2[P_T]$. At $\bar{\nu} = 1$, $[P] = [PL_2] = 0.25[P_T]$ and $[PL_1] = 0.5[P_T]$.

(3) Divalent Protein and Monovalent Cell Surface Ligand. Let us define the following terms: C = number of cells/liter, s = number of sites/cell, and V^* = surface volume of a shell surrounding the cell. The concentration of any surface-bound species in the shell of volume V^* averaged over all cells is designated by square brackets with an asterisk, in moles/liter, and the concentration of any species referred to the *total volume* of solution is designed by square brackets alone. Then

$$[L]^* = [L]/CV^* \qquad [PL_1]^* = [PL_1]/CV^* \tag{11}$$
$$[PL_2]^* = [PL_2]/CV^*$$

The total concentration of surface-bound ligand is

$$[L_T]^* = [L]^* + [PL_1]^* + 2[PL_2]^* = s/V^*N \tag{12}$$

in molar units where N is Avogadro's number. Then

$$[L_T] = [L_T]^*CV^* = Cs/N \tag{13}$$

Consider now the case of a divalent protein in solution binding to freely mobile receptor sites on a cell surface:

$$P + L^* \rightleftharpoons PL_1^* \tag{14}$$
$$PL_1^* + L^* \rightleftharpoons PL_2^*$$

Using the definitions and equations given above

$$K_1 = \frac{[PL_1]^*}{[P][L]^*} = \frac{[PL_1]}{[P][L]}$$
$$K_2 = \frac{[PL_2]^*}{[PL_1]^*[L]^*} = \frac{[PL_2]CV^*}{[PL_1][L]} \tag{15}$$

The first reaction occurs by means of collision of the protein with a cell in the bulk solution, and the expression for K_1 in terms of bulk concentrations is the same as it would be for a reaction taking place between soluble species (see eq 7). This is not true for K_2 which refers to a reaction taking place entirely on the cell surface where the effective concentrations of ligand and protein differ from those in the bulk solution.

Table I: Radii of Different Cell Types

	$2r$ (cm)	A (cm^2)
small lymphocyte	7×10^{-4}	1.54×10^{-6}
lymphoblast	2×10^{-3}	1.26×10^{-5}
mast cell	1×10^{-3}	3.14×10^{-6}
neuronal cell body from lobster ganglion	2×10^{-2}	1.26×10^{-3}

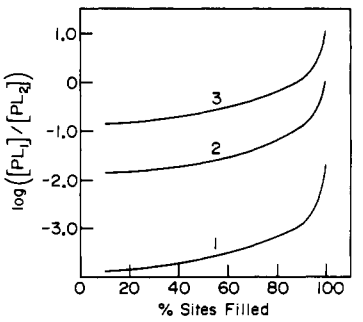

FIGURE 1: Dependence of $[PL_1]/[PL_2]$ on percent of sites filled (eq 18), $K_2 = 10^6$, $\delta A = V^* = 2 \times 10^{-15}$ L. (1) $s = 10^7$ sites/cell, (2) $s = 10^5$ sites/cell, (3) $s = 10^4$ sites/cell.

We can express the total protein concentration in bulk solution as

$$[P_T] = [P] + [PL_1] + [PL_2] = [P] + K_1[P][L] + \frac{K_1K_2[P][L]^2}{CV^*} \tag{16}$$

The ratio of total moles of bound ligand to total moles of protein, $\bar{\nu}$, is

$$\bar{\nu} = \frac{[PL_1] + 2[PL_2]}{[P_T]} = \frac{K_1[L] + 2K_1K_2[L]^2/CV^*}{1 + K_1[L] + K_1K_2[L]^2/CV^*} \tag{17}$$

V^* for a particular cell is defined by the cell surface area A and an arbitrarily chosen surface layer thickness, δ. For a spherical cell of radius r, $A = 4\pi r^2$ and $V_1 = (4/3)\pi r^3$. The volume of this cell with radius $r + \delta$ is $V_2 = (4/3)\pi(r + \delta)^3$. Therefore, $V_2 - V_1 = V^* = (4/3)\pi(r^3 + 3r^2\delta + 3r\delta^2 + \delta^3 - r^3)$, and since δ is small relative to r, $V^* = 4\pi r^2\delta = A\delta$. (Typically, $2r$ for a cell ranges from 7×10^4 to 20×10^4 Å as is shown in Table I. No cell, of course, has a perfectly smooth surface nor is perfectly spherical; so in general our V^* is an underestimate. A reasonable value of δ is of the order 100 Å.)

At this point we should consider the relative contribution of the forms $[PL_1]$ and $[PL_2]$ to a binding isotherm described by eq 17. From eq 13 and 15 and using the relationship $[L] = Cs/N - Cs_{filled}/N$ we observe that

$$\frac{[PL_1]}{[PL_2]} = \frac{CV^*}{K_2[L]} = \frac{V^*N}{K_2(s - s_{filled})} \tag{18}$$

At low protein concentrations and large excess of ligand such that $s - s_{filled} \simeq s$, $[PL_1]$ is small relative to $[PL_2]$, but at high $[P_T]$ and limiting $[L_T]$, $[PL_1]$ becomes significant. This is demonstrated in Figure 1.

If $[PL_2] >> [PL_1]$, eq 17 becomes

$$\bar{\nu} = \frac{2K_1K_2[L]^2/CV^*}{1 + K_1K_2[L]^2/CV^*} \tag{19}$$

If $[PL_1] >> [PL_2]$, eq 17 becomes

$$\bar{\nu} = \frac{K_1[L]}{1 + K_1[L]} \tag{20}$$

Note that in eq 19 $\bar{\nu}$ depends upon $[L]^2/C$ and not upon [L] only. The consequence of this relationship is that a binding isotherm of $\bar{\nu}$ vs. log [L] will differ when $[P_T]$ is constant and C is varied from that when C is constant and $[P_T]$ is varied. The appropriate relationship when $[PL_2]$ is the principal species is $\bar{\nu}$ vs. $[L]^2/C$.

In the case of soluble divalent protein and monovalent antigen bound to the cell surface (eq 17), we cannot further simplify the relationships we have derived since it is not legitimate to set $K_1 = 4K_2 = 2K$ (as we did in the previous section for k_1 and k_2). Orientation factors and restriction of motion of both surface ligand and bound protein will usually act to reduce the ratio of K_2 to K_1 below the statistical value of 0.25. The absolute ratio cannot be predicted a priori.

At $\bar{\nu} = 1$, the midpoint of the binding curves, $[L]_{\bar{\nu}=1}$ is a function of several parameters as long as $[PL_2]$ is a significant species:

$$[L]_{\bar{\nu}=1} = \left(\frac{CV^*}{K_1K_2}\right)^{1/2} = \left(\frac{CA\delta}{K_1K_2}\right)^{1/2} \tag{21}$$

Since $A\delta$ cannot be precisely determined, these data would not provide a measure of K_1K_2. In any case, when the ligand is surface bound, it is usually *not* possible to determine $\bar{\nu}$ and [L] experimentally, and one is limited to a measurement of free protein [P] as a function of C.

From eq 16

$$\frac{[P]}{[P_T]} = \frac{1}{1 + K_1[L] + K_1K_2[L]^2/CV^*} \tag{22}$$

If the experiment is designed such that total ligand is in large excess and the protein concentration is low, $[L] \simeq [L_T] = Cs/N$, and

$$\frac{[P]}{[P_T]} = \frac{N^2V^*}{N^2V^* + NV^*K_1Cs + K_1K_2Cs^2} \tag{23}$$

At the midpoint of $[P] = {}^1/_2[P_T]$

$$C_{1/2} = \frac{N^2V^*}{NV^*K_1s + K_1K_2s^2} = \frac{N^2A\delta}{NA\delta K_1s + K_1K_2s^2} \tag{24}$$

The concentration of cells at which half the protein is bound depends not only on K_1 and K_2 but also on the number of sites and surface volume.

Equations 23 and 24 apply only when the ligand is present in large excess relative to protein. If the experiments are conducted under conditions where this assumption does not hold, it is necessary to find a suitable expression for [L] in terms of constant terms and the variable parameter, C.

The following expression gives the relationship between [L], C, and $[P_T]$:

$$[L]^3K_1K_2 + [L]^2(CV^*K_1 - K_1K_2[L_T] + 2K_1K_2[P_T]) + [L](CV^* + CV^*K_1[P_T] - CV^*K_1[L_T]) - [L_T]CV^* = 0 \tag{25}$$

It is apparent that a tractable relationship between $[P]/[P_T]$ and C cannot be obtained by our usual procedure of substituting an explicit expression for [L] in eq 22.

(4) Comparison of Amount of Surface Antigen on Different Cell Types Using the Same Antibody Preparation. In immunological research it is often desirable to compare relative amounts of a specific antigen on the surface of different cell types (e.g., Williams et al., 1977; Stohl & Gonatas, 1977). These studies are usually carried out by determining the relative value $[P]/[P_T]$ as a function of C using identical antibody preparation. A comparison of the midpoints of a series of such binding data using different cell types is often implied to be a measure of relative amounts of the specific antigen being investigated.

Table II: Comparisons of Thy-1 Antigen on Different Tissues[a]

	$\frac{C_{1/2}}{C_{1/2}'}$	$\frac{s'}{s}\left(\frac{A}{A'}\right)^{1/2}$	fraction[b] of labeled cells
mouse thymus	1.0	1.0	0.95
mouse lymph node	0.14	0.38	0.55
mouse spleen	0.07	0.27	0.30
rat thymus	0.90	0.95	0.92
rat lymph node	0.007	0.08	0.05
rat spleen	0.035	0.19	0.15

[a] Williams et al. (1977). [b] Autoradiography using ^{125}I label.

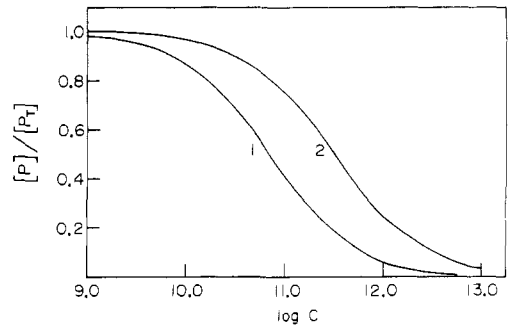

FIGURE 2: Dependence of $[P]/[P_T]$ vs. C on cell radius (eq 22), $s = 10^5$ sites/cell, $K_1 = 2 \times 10^6$ L mol^{-1}, $K_2 = 0.5 \times 10^6$ L mol^{-1}. (1) $\delta A = V^* = 2 \times 10^{-15}$ L, (2) $\delta A = V^* = 1 \times 10^{-14}$ L.

Equation 23 demonstrates that in fact the ratio of midpoints is given by

$$\frac{C_{1/2}}{C_{1/2}'} = \left(\frac{N\delta A'K_1s' + K_1K_2(s')^2}{N\delta AK_1s + K_1K_2s^2}\right)\frac{A}{A'} \tag{26}$$

at $[L] \simeq [L_T]$ and with identical association constants since the antibody and ligand are the same in both cell systems.

If $[PL_1] << [PL_2]$, as would be expected at low protein to ligand ratios ($[L] \simeq [L_T]$), eq 26 reduces to

$$\frac{C_{1/2}}{C_{1/2}'} = \frac{A(s')^2}{A'(s)^2} \tag{27}$$

and we still do not have a direct measurement of relative values of s since A is not known for the cell systems being investigated. Note also that even in the fortuitous event that $A = A'$, $C_{1/2}/C_{1/2}'$ is the ratio of the *square* of the number of sites/cell. Table II presents data from Williams et al. (1977), demonstrating a further problem in this type of analysis—a nonhomogeneous cell system. Note that when the correct relationship between midpoints and sites is used (eq 27) the agreement between binding data and number of cells actually binding antibody is reasonable. This result suggests that those cells which are labeled in the nonhomogeneous populations may contain the same number of antigenic sites/surface area as the homogeneous thymus cells.

Figure 2 shows binding data which would be obtained when identical antibody interacts with the same ligand on cells differing by a factor of 2.2 in radius but having the same absolute number of sites.

(5) Comparison of Different Antibody Preparations Using the Same Cell System. Binding data for *different* antibody

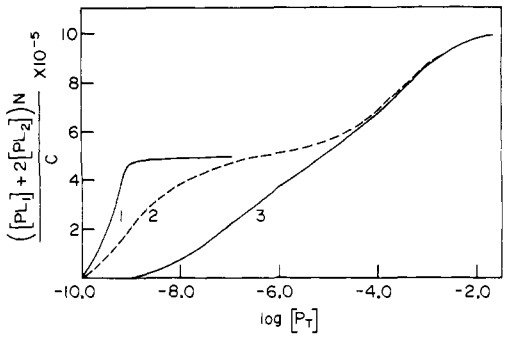

FIGURE 3: Saturation binding of antibody to a cell surface, $s = 10^6$ sites/cell, $C = 10^9$ cells/L, $V^* = \delta A = 2 \times 10^{-15}$ L. (1) $K_1 = 2 \times 10^8$, $K_2 = 0.5 \times 10^8$ L mol^{-1}, (2) $K_1 = 2 \times 10^6$, $K_2 = 0.5 \times 10^6$ L mol^{-1}, (3) $K_1 = 2 \times 10^5$, $K_2 = 0.5 \times 10^5$ L mol^{-1}.

preparations to the *same* cell systems is often used to compare relative affinities. Again using eq 23

$$\frac{C_{1/2}}{C_{1/2}'} = \frac{N\delta AK_1' + K_1'K_2's}{N\delta AK_1 + K_1K_2s} \tag{28}$$

when $[L] \simeq [L_T]$. At low protein concentration and low site occupancy, $[PL_2]$ is favored over $[PL_1]$, and eq 28 reduces to

$$C_{1/2}/C_{1/2}' = K_1'K_2'/K_1K_2 \tag{29}$$

so that the ratio of midpoints provides a measurement of relative affinities of different antibody preparations as long as the ligand cell system is identical in all experiments.

(6) *Determination of Total Sites/Cell.* The most desirable procedure for determining s is by means of eq 6 which is applicable if the binding of Fab is measured as a function of free protein. If intact antibody or $(Fab)_2$ is used, both $[PL_1]$ and $[PL_2]$ can be present on the cell surface and a direct binding measurement of s is ambiguous. Figure 3 shows three different results which can be obtained in the same system depending upon the magnitude of K_1 and K_2. Extrapolation to high values of $[P_T]$ will lead to a limiting value of s or $s/2$ depending upon K_1 and K_2.

There is another way of determining s. If $[PL_2] >> [PL_1]$, we can express [L] in terms of $[L_T]$, $[P_T]$, and [P] because there is only one species with bound L.

Equation 22 is rewritten as

$$\frac{[P]}{[P_T]} = \frac{CV^*}{CV^* + K_1K_2[L]^2} = \frac{CV^*}{CV^* + K_1K_2\left(\frac{Cs}{N} - 2[P_T] + 2[P]\right)^2} \tag{30}$$

If we determine [P] as a function of $[P_T]$ and C, we can determine s directly from the following equation which is simply an algebraic rearrangement of eq 30. When $[P] = {}^1/_2[P_T]$

$$[P_T] - \frac{C_{1/2}s}{N} \equiv \left(\frac{C_{1/2}V^*}{K_1K_2}\right)^{1/2} \tag{31}$$

A plot of $[P_T]$ vs. $C_{1/2}$ will be linear with an intercept indistinguishable from zero if K_1K_2/V^* is large relative to C. The slope of this line corresponds to s/N.

Equation 30 can be solved and rearranged for any value of $[P]/[P_T]$. If the assumptions regarding the predominance of $[PL_2]$ and the relationship between K_1K_2/V^* and C are valid, s must be demonstrated to be independent of $[P_T]$ or $[P]/[P_T]$. This procedure was used by Morris & Williams (1977) to determine the number of antigenic sites on rat thymocytes

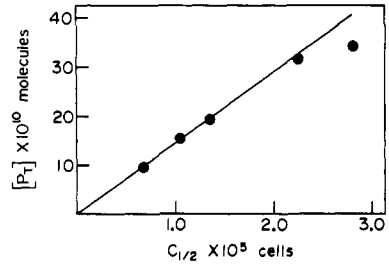

FIGURE 4: Dependence of $C_{1/2}$ on $[P_T]$ for Thy-1 antigen on thymocytes (Morris and Williams, 1977).

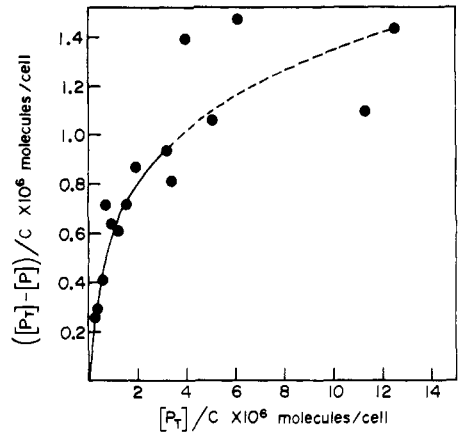

FIGURE 5: Bound antibody per cell as a function of total antibody: Thy-1 antigen on thymocytes (Morris and Williams, 1977).

which recognized anti-Thy-1 antibody. Figure 4 is the plot of their data at $[P]/[P_T] = {}^1/_2$. Note that the two upper points fall off the straight line. The value of s obtained from the slope is 1.44×10^6 sites/cell. If all of the data reported by Morris & Williams are plotted as $([P_T] - [P])/C$ vs. $[P_T]/C$, we obtain Figure 5 which clearly indicates that the total amount of antibody which can be bound at high $[P_T]/C$ is approaching 1.44×10^6 molecules/cell. It appears that at high levels of site occupancy, $[PL_1]$ becomes the predominant species as was predicted theoretically in section 3. (The authors, in fact, misinterpreted their binding data as representing a maximum of 700 000 molecules of antibody bound per cell.)

(7) *Comparison of Solubilized Antigen and Cell-Surface Antigen.* Two types of experiments have been reported (Williams et al., 1977) comparing solubilized antigen with that bound to a cell surface. In the first case, detergent is added to a known number of cells at a concentration sufficient to form lipid–detergent micelles and antigen–detergent complexes. If the latter contain *monomeric* antigen, the binding of antibody obeys eq 9 and 10. If the antigen is in large excess such that $[L] \simeq [L_T] = Cs/N$ eq 10 becomes

$$\frac{[P]}{[P_T]} = \frac{N^2}{N^2 + 2KCsN + K^2C^2s^2} \tag{32}$$

Note that this equation differs from that for cell-surface-bound ligand, eq 23, in that the latter contains V^*. Thus, binding curves for these two systems will generally differ even if the binding affinities are identical and identical amounts of ligand are present. This type of experiment does not provide information regarding possible detergent activation or inactivation of the antigenic activity.

In order to discuss this in more detail, we need to explore the relationship between k_1 and k_2 and K_1 and K_2 (eq 7 and 15). Equilibrium constants are functions of translational, vibrational, rotational, and electronic energies of the molecules involved in the reaction [for a complete discussion, see Hill

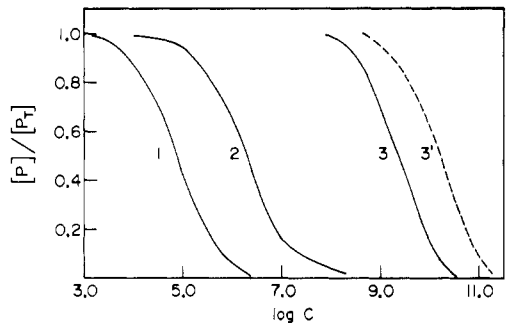

FIGURE 6: $[P]/[P_T]$ as a function of C (eq 23 and 32), $s = 10^5$ sites/cell, $V^* = \delta A = 2 \times 10^{-15}$ L. (1) $K_1 = 2 \times 10^9$, $K_2 = 0.5 \times 10^9$ L mol^{-1} (cell-surface antigen), (2) $K_1 = 2 \times 10^9$, $K_2 = 2 \times 10^7$ L mol^{-1} (cell-surface antigen), (3) $K_1 = 2 \times 10^9$, $K_2 = 0.5 \times 10^9$ L mol^{-1} (soluble antigen). [P] only determined by assay procedure. (3') Same as 3 but both [P] and $[PL_1]$ determined by assay procedure.

(1960)]. It is intuitively obvious that translational and rotational energies must differ for molecules in solution and those attached to cell surfaces. Hence K_1 and K_2 cannot be predicted a priori even when k_1 and k_2 are known. As a specific example of the problems arising in a comparison of soluble and cell-surface antigen, let us assume as a first approximation that $K_1 = k_1$. In a system containing 10^9 cells/L with an assumed V^* approximately 10^{-15}L, K_2 would need to be approximately $10^{-6}k_2$ if the binding curves for soluble and cell-bound antigen (eq 23 and 32) are to be nearly superimposable. Such a large decrease in the second binding constant is difficult to justify.

Figure 6 presents hypothetical binding data for the interaction between antibody and soluble or membrane-bound form of an antigen assuming (curves 1 and 3) $K_1 = k_1$ and $K_2 = k_2$ and also (curves 2 and 3) $K_1 = k_1$ and $K_2 = (1/100)k_2$. The difference between the isotherms increases as the association constants increase due to the smaller contribution of $[PL_2]$-(membrane form) at high values of K_1 and K_2. (An additional problem in a comparison of this type is discussed more fully below and relates to the experimental procedure used to determine $[P]/[P_T]$.)

The ratio of midpoints for binding data of $[P]/[P_T]$ vs. C for solubilized antigen and cell-surface antigen is (from eq 23 and 32)

$$\frac{C_{1/2}(\text{soluble})}{C_{1/2}(\text{cell})} = \frac{0.414N}{(k_1k_2)^{1/2}s}\left(\frac{NA\delta K_1 s + K_1K_2s^2}{N^2A\delta}\right) \tag{33}$$

where C (soluble) refers to the number of cells dissolved by the detergent. Since $k_1 = 4k_2$, if $k_1 = K_1$ and $k_2 = K_2$

$$\frac{C_{1/2}(\text{soluble})}{C_{1/2}(\text{cell})} = 0.818\left(1 + \frac{K_2 s}{NA\delta}\right) \tag{34}$$

If $[PL_2] >> [PL_1]$, eq 34 reduces to

$$\frac{C_{1/2}(\text{soluble})}{C_{1/2}(\text{cell})} = 0.818\frac{K_2 s}{A\delta N} \tag{35}$$

Thus, even if the amount of antigen in both soluble and membrane-bound forms is identical, the binding data for these two systems cannot be compared in any meaningful way. The relative positions of two such isotherms are dependent on several parameters, and even reasonable assumptions about the relative affinities are not sufficient to overcome the problem since the factor $V^* = A\delta$ remains in the equation.

A second type of experiment involves the binding of purified detergent solubilized antigen to antibody. In this case, $[P]/[P_T]$ is determined as a function of total antigen added. Equation 10 applies if $[L] \simeq [L_T]$ as

$$\frac{[P]}{[P_T]} = \frac{1}{(1 + K[L_T])^2} \tag{36}$$

From eq 10 and 24, we obtain the ratio of midpoints for this system and cell-bound antigen as

$$\frac{[L_T]_{1/2}(\text{soluble, pure})}{C_{1/2}(\text{cell})} = \frac{0.414(NK_1sA\delta + K_1K_2s^2)}{N^2KA\delta} \tag{37}$$

Thus, a comparison of this type by means of binding data does not provide a measure of the number of sites/cell as has been suggested even if $K_1 = k_1$, $K_2 = k_2$, and $[PL_2] >> [PL_1]$. Even with these simple simplications, eq 37 becomes

$$\frac{[L_T]_{1/2}}{C_{1/2}} = \frac{0.414s^2}{N^2A\delta} \tag{38}$$

Removal of detergent from purified antigen preparations obtained from membranes usually leads to aggregation of the antigen. In this situation one has a multivalent ligand in solution reacting with a divalent antibody. The ligand can no longer be treated as we did in section 3 and binding data for this system *cannot* be compared in any manner with the systems we have thus far described.

Thus far we have not discussed experimental procedures for determining bound and free antibody. If the system is antibody–cell-surface ligand, the cells with bound antibody can be separated from the bulk solution by centrifugation or filtration and the supernatant assayed directly for [P]. However, in the case of soluble antigen in detergent micelles no such means of separation exists. It has been suggested that the total solution can be assayed for unbound antibody by adding glutaraldehyde-fixed cells containing the membrane-bound antigen (Williams, 1977). This amounts to a competition experiment described by the following equilibria:

$$\begin{gathered}\text{Ab} + \text{Ag} \rightleftharpoons \text{AbAg}_1 \qquad \text{AbAg}_1 + \text{Ag} \rightleftharpoons \text{AbAg}_2 \\ \text{Ab} + \text{cells} \rightleftharpoons \text{Ab–cells}\end{gathered} \tag{39}$$

All three equilibria are assumed to be irreversible under the conditions of the experiment. Even if pseudoirreversibility can be demonstrated, a serious problem arises in that one does not know a priori whether the cells can interact only with free antibody or also with that with one site occupied by soluble ligand, $AbAg_1$. Figure 6 (curves 3 and 3') demonstrates the difference in binding curves obtained with this system in the two cases of only free antibody detected by the assay procedure or both free antibody and that with one occupied site detected.

Discussion

We have described the thermodynamics of interaction between divalent protein in solution and monovalent ligand bound to, but mobile in, a cell surface. Two major simplifying assumptions have been used: (1) a homogeneous population of cells in terms of surface area and number of ligand molecules/cell and (2) a homogeneous population of divalent antibody. We have shown that in most cases experimental data are not interpretable unambiguously in even this simplified system due to lack of priori knowledge of V^*, $K_1 = f(K_2)$, and hence of $[PL_1]/[PL_2]$. It is particularly important to realize that the straightforward relationships obtained in section 2 for divalent protein and monovalent ligand (both in solution) are the mathematical result of a known statistical relationship between k_1 and k_2. Such a relationship is not predictable from first principles between K_1 and K_2.

Attempts to compare relative amounts of a specific surface antigen on different cell types by measuring the number of cells required to bind 50% of added antibody are in fact feasible

only when antigen is present in large excess, when $[PL_2] >> [PL_1]$, and when the cell population is homogeneous. Even in this specific and restricted case, one measures the ratio between the *square* of the number of sites per surface area of the cell—not the ratio of the absolute number of sites.

A comparison of relative antibody affinities using the same cell system is valid as long as $[PL_2]$ is the predominant species.

The determination of the total number of antigenic sites per cell is subject to a possible error by a factor of 2 depending upon the relative association constants K_1 and K_2 as well as the range of $[P_T]/C$ used in the experiment. Monovalent Fab binding to cell surfaces provides an unequivocal result. However, experimentally this can be done only if the antibody affinity is high and the number of antigenic sites per cell is at least 10^5. At low antibody affinity and s values, the number of cells/liter required for measurable interaction becomes prohibitively high.

Finally, all attempts to compare soluble and membrane-bound forms of an antigen as described by Williams (1977) are shown in section 7 to be fundamentally invalid.

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References

Bell, G. I. (1974) *Nature (London) 248*, 430.
Bell, G. I., & De Lisi, C. (1974) *Cell. Immunol. 10*, 415.
De Lisi, C., & Perelson, A. (1976) *J. Theor. Biol. 62*, 159.
Dembo, M., & Goldstein, B. (1978) *J. Immunol. 121*, 345.
Hill, T. L. (1960) *An Introduction to Statistical Thermodynamics*, Addison-Wesley, Reading, MA.
Morris, R. J., & Williams, A. F. (1977), *Eur. J. Immunol. 7*, 360.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
Steinhardt, J., & Reynolds, J. A. (1969), *Multiple Equilibria in Proteins*, Academic Press, New York.
Stohl, W., & Gonatas, N. K. (1977), *J. Immunol. 119*, 422.
Tanford, C. (1961), *Physical Chemistry of Macromolecules* Wiley, New York.
Williams, A. F. (1977) *Contemp. Top. Mol. Immunol. 6*, 81.
Williams, A. F., Barclay, A. N., Letante-Muirhead, M., & Morris, R. J. (1977) *Cold Spring Harbor Symp. Quant. Biol. 41*, 51.

Changes in Poly(adenylic acid) Polymerase Activity during Sea Urchin Embryogenesis†

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ABSTRACT: Between fertilization and the two-cell stage there is a doubling of the poly(adenylic acid) [poly(A)] content of sea urchin embryos. This net increase in poly(A) is due to cytoplasmic polyadenylation of stored underadenylated messenger RNA molecules. The present work was initiated to determine the mechanisms responsible for the changes in the rate of poly(A) synthesis during early sea urchin development. The total poly(A) polymerase activity in *Strongylocentrotus purpuratus* is constant during development, from the unfertilized egg until the prism stage. As development proceeds, however, there is a rearrangement in the subcellular localization of the enzyme. In the unfertilized egg, the enzyme activity is almost entirely localized in the 100000g supernatant fraction. As embryogenesis proceeds, there is a progressive increase in the percent of enzyme activity which is associated with the nuclear fraction, along with a concomitant decrease in the supernatant activity. A substantial proportion of the poly(A) polymerase activity is present in enucleated as well as nucleated merogons prepared from *S. purpuratus* eggs indicating that the soluble poly(A) polymerase activity observed during early development is due to an actual cytoplasmic localization rather than nuclear leakage. The post-fertilization increase in polyadenylation, therefore, need not be accomplished by de novo synthesis of poly(A) polymerase.

Fertilization or artificial activation of sea urchin eggs initiates increases in protein, DNA, and RNA synthesis (Gross, 1967). During the period of increasing protein synthesis, the poly(A)[1] content of the embryo increases to more than twice the level present in unfertilized eggs (Slater et al., 1972; Wilt, 1973). This net increase in polyadenylation occurs on underadenylated messenger RNAs stored in the egg cytoplasm and is essentially complete by the time of the second cleavage (Wilt, 1973; Slater et al., 1973; Slater & Slater, 1974). The fact that polyadenylation occurred to the same extent in parthenogenetically activated enucleated egg fragments (Wilt, 1973) implied that a cytoplasmic mechanism for polyadenylating messenger RNA exists. Since this demonstration, nonmitochondrial cytoplasmic polyadenylation has been detected in mammalian cell lines (Brawerman & Diez, 1975; Sawicki et al., 1977), and important roles for the poly(A) segment in controlling messenger RNA stability in the cytoplasm have been suggested (Nudel et al., 1976; Huez et al., 1978).

The mechanisms by which protein, DNA, RNA, and poly(A) syntheses increase after fertilization are not known.

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[1] Abbreviations used: poly(A), poly(adenylic acid); poly(G), poly(guanylic acid); poly(C), poly(cytidylic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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