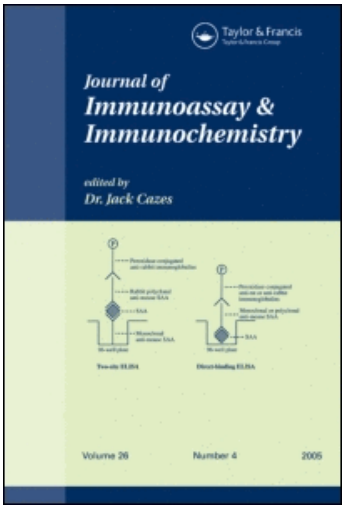

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Butler, John E.(2000) 'Enzyme-Linked Immunosorbent Assay', Journal of Immunoassay and Immunochemistry, 21: 2, 165 — 209
To link to this Article: DOI: 10.1080/01971520009349533
URL: http://dx.doi.org/10.1080/01971520009349533

JOURNAL OF IMMUNOASSAY, 21(2&3), 165–209 (2000)

Enzyme-Linked Immunosorbent Assay

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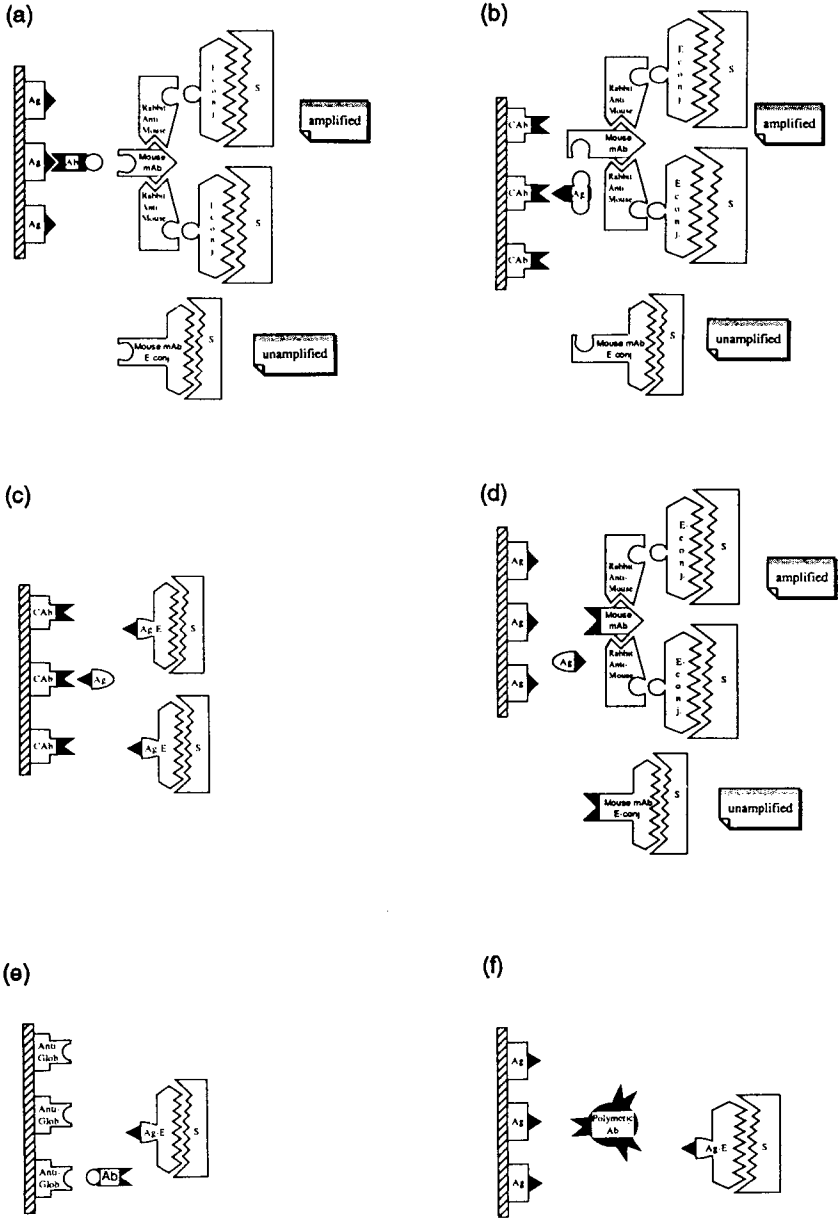
I. INTRODUCTION

A. History

The acronym for enzyme-linked immunosorbent assay (ELISA) has become a standard abbreviation in many scientific journals. ELISAs are immunoassays in which one reactant is immobilized on a solid phase and the "signal generator" or "reporter," is an enzyme. This definition includes enzyme immunoassay (EIA) which like classical radioimmunoassay (RIA), is competitive in design, but employs an enzyme reporter instead of a radionuclide. ELISAs and EIAs, which consist of both a solid and a fluid phase, are designated *heterogeneous EIAs* (HetEIA). *Homogeneous enzyme immunoassays* (HomEIA) do not involve a solid phase and are often not included under the ELISA umbrella, although they will be briefly discussed in this chapter. The HetEIA is often treated under the category of *solid-phase immunoassay* (SPI) because assays done on a solid phase have more in common with each other than they do with assays that share a common reporter system (i.e., an enzyme). ELISA and SPI are performed on a variety of solid phases ranging from smooth polystyrene to membranous nitrocellulose (NC) and nylon. The term ELISA was coined by Engvall and Perlmann in 1971 for a noncompetitive HetEIA configuration, whereas EIA historically denoted a competitive one. These acronyms, as well as EMI (enzyme-mediated immunoassay) and others, are often used interchangeably in the literature.

Solid-phase immunoassays and ELISA have technically simplified antigen quantitation and antibody detection. They are the basis for most modern immunodiagnostic tests for acquired immunodeficiency syndrome (AIDS), hepatitis, allergies, and many other human and animal diseases. Before 1970, nearly all immunodiagnostic tests were based either on fluid phase or cellular interactions of antigens and antibodies (e.g., agglutination, precipitation, and complement fixation). Also before that time, immunological tests for quantifying the levels of small molecules used radioisotopes as reporters and involved tedious procedures for the separation of the bound and free reactants. The observation that proteins are spontaneously adsorbed to hydrophobic surfaces (Catt and Tregear, 1967), led to the development of contemporary SPI, which technically simplified immunoassays and permitted them to be easily automated. Add to this development the use of enzymes as alternatives to radioisotopes, and you describe "immunoassay for the common man" (i.e., ELISA).

Reprinted from *Immunochemistry*, C. J. van Oss and M. H. V. van Regenmortel, eds., Marcel Dekker, Inc., New York, 1994, 759–803.



B. General Principles and Nomenclature

The fundamental principle of ELISA, from which the name is derived, is the use of an enzyme to deliver a signal that a particular antigen–antibody reaction has occurred and to what extent. Enzymes are highly specific, and their catalytic properties can enhance a nonenzymatic reaction a billionfold (Fersht, 1977). Furthermore, enzyme signals, unlike those of radionuclides or fluorescent compounds, increase with time by continuing to turnover more substrate. The use of various enzyme-amplification circuits (see Sec. II) can achieve signal levels two to three orders of magnitude greater than those obtained using radionuclides.

Figures 1 and 2 illustrate (1) the various configurations used in ELISAs and (2) the factors that determine specificity and nonspecific binding (NSB). All ELISA configurations are composed of three components: the *capture* system, the *analyte* [the substance that the assays are designed to measure; either antibody (Fig. 1a) or antigen (Fig 1b)], and the *detection* system. In each the analyte must be the one in limiting amount; both the solid-phase capture reagent and the detection system must be present in *functional molar excess* (FMX). In a direct competitive assay (see Fig. 1c), a known amount of labeled analyte itself provides the detection system. In this sytem, the unlabeled analyte in the test sample must be in lowest amount. The terms ligand and receptor are often used interchangeably with antigen and antibody in immunochemistry. In this chapter, the solid-phase capture reagent (or reactant) is always regarded as the receptor or capture reagent, regardless of whether it is antibody or antigen. The term FMX is a theoretical value which recognizes that seldom in practice are 100% of the reactant molecules functionally active, thus necessitating addition of the reactant at a concentration greatly exceeding its actual molar excess.

Figure 1a illustrates the analyte-limiting principle in the detection of human antibodies to an immobilized (solid-phase) antigen in a noncompetitive configuration. The human antibodies (indicated as a filled small symbol) are the reaction-limiting analyte. Once these antibodies are bound, the fluid phase, containing the irrelevant serum protein and free antibody at equilibrium, is removed. The remaining bound human antibodies are then detected by a multistage system, first involving mouse monoclonal (mAb) to human IgG, followed by a biotinylated rabbit antimouse IgG, then a streptavidin–alkaline phosphatase *conjugate* (E-conj), and finally the substrate *p*-nitrophenyl phosphate (pNPP). The antibody-containing serum is diluted (i.e., "titered") until the antibodies to be measured (analyte) are indeed reaction-limiting. Such proper dilutions are now within the working range (see later) of the assay.

Another basic principle is that reagents that are added progressively *after the analyte* must each be added in FMX in relation to the reactant *bound* during the assay step immediately before. This is illustrated in Fig. 1a, b, and d by the use of progressively larger symbols for the reactants in the amplification chain. This means that time and reagents will be required to titer each component in the chain against the previous one to assure FMX. Fortunately, the same multistage detection system can often be used in a variety of tests. For example, the detection system depicted in Fig. 1a can also be used to measure human antibodies to other isotypes and subclasses merely by inserting a mouse mAb with specificity for the antibody isotype to be measured.

Figure 1 ELISA principles and configurations: six of the more popular ELISA configurations are illustrated (a–f). Each is described in the text. In each configuration, the smallest symbol is the one that limits the reaction and is the analyte being measured. The multireactant system depicted is only one type of *amplified* system used in ELISA (see text). The multireactant systems depicted best illustrate the progressive functional molar excess of the more distal reactants, which is a principle common to all configurations; this is illustrated by their increasing size. ◀, primary antibody–antigen interactions (both paratope and epitope are dark-shaded). Other specific interactions are depicted by conventional lock-and-key cartoons.

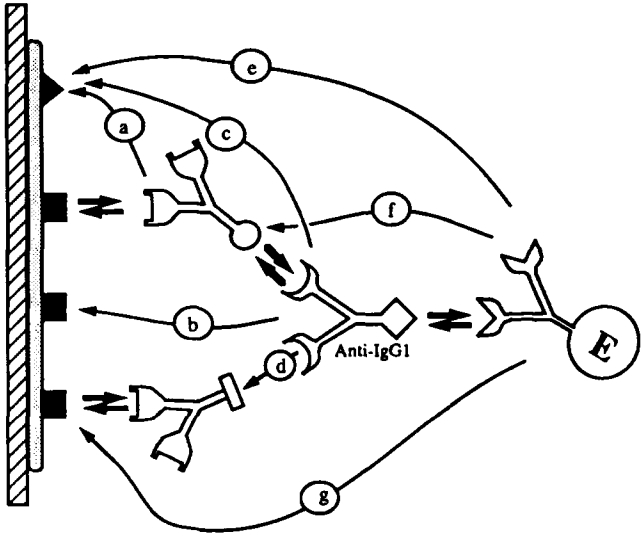


Figure 2 Short-circuiting produces nonspecific binding (NSB) in ELISA: Antibodies are depicted as bivalent and as Y-shaped molecules. The shape of their Fabs and Fcs are designed to depict their paratope and epitope specificities. The solid, unidirectional arrows with arabic letters depict the various short circuits possible. Double, reciprocal arrows depict the desired or normal reaction pathway. The various short circuits illustrated are as follows: a, primary antibody (analyte) cross-reacts with a second undesirable solid-phase epitope; b, secondary antibody (part of detection system) recognizes the primary antigen on the solid phase; c, secondary antibody recognizes the second undesirable epitope on the solid phase; d, secondary antibody cross-reacts with a primary antibody of a different isotype than that for which the assay was designed to measure; e, the tertiary antibody (or reactant that carries the reporter enzyme) recognizes an undesirable solid-phase epitope; f, the tertiary reactant recognizes and binds the primary antibody (analyte) and thus bypasses the secondary antibody; g, the tertiary reactant binds to the solid phase itself. The latter situation could theoretically occur with any of the reactants.

The so-called dynamic or working range of the assay is largely dependent on the extent of the FMX of the solid-phase capture reagent. If the surface of a microtiter well contains 500 ng of capture antibody (CAb; see Fig. 1b), then the theoretical range is from 0 to 500 ng of antigen, provided (1) all 500 ng of immobilized reactant is functional; (2) one molecule of CAb can capture one molecule of antigen; (3) the equilibrium constant (K_{eq}) is such that when 500 ng of antigen is added, 500 ng is bound, and when 1 ng is added, 1 ng is bound; and (4) when 1 ng is bound, it can be detected. In practice, usually none of these assumptions are met. Rather, less than 100% of the immobilized reactant is functional (see Sec. II), the K_{eq} does not permit 100% capture of the analyte, and the detection system does not permit 1 ng to be recognized. More realistically, the working range of such an assay might instead be 10–200 ng because (1) the detection system gives no signal significantly above background when less than 10 ng of analyte is captured; (2) only 20% of the solid-phase reactant is functional; and (3) only one-half of the added analyte is captured at equilibrium. The dynamic range can be increased by (1) lowering the detection limit through signal amplification; (2) increasing the concentration of the *functional* solid-phase reactant through decreasing its loss of activity using alternative methods of immobilization (see

Sec. II); (3) immobilizing *more* capture reagent by increasing the surface area of the solid phase without increasing the working "reaction volume" of the assay; and (4) using an interaction of lower K_{eq} for the primary reaction to extend the working range to higher concentrations. Unfortunately, detection of low concentrations of analyte will suffer from the last manipulation. Thus, some investigators use blended mAbs of varying affinity to broaden the dynamic range in both directions (Lindmo et al., 1990; see Fig. 7c).

Detectability of the analyte (i.e., *sensitivity*) is inversely proportional to the number of analyte molecules needed to produce a signal above background. Sensitivity is often increased through amplification; Fig. 1a, b, and d illustrate use of simple detection system as well as a multistage amplification system for detecting the same analyte. There are two general methods for achieving amplification. One depends on increasing the ratio of active enzymes molecules to analyte (e.g., see Fig. 1a, b, and d) and the *amplified ELISA* (Butler et al., 1978b). The second method involves increasing the signal produced when one enzyme molecule converts one molecule of substrate. The latter involves the use of enzyme cascades, cofactor recycling, and the generation of fluorescent and chemiluminescent end products (reporter systems are the topic of Sec. III).

The maximum sensitivity of ELISAs and other heterogeneous immunoassays has been shown by Ekins and co-workers (Ekins, 1983, 1991) to be best determined from the precision of the zero dose (without analyte) response, that is

$$k(\Omega)/K = 0.5 \times 10^{-5} \text{ mol/L} \quad (1)$$

In this equation, k = fractional nonspecific binding of antibody (10^{-14}), $K = K_{eq}$ of the interaction ($2 \times 10^{10}\ M^{-1}$), and Ω = the relative error at zero dose (e.g., nonspecifically bound antibody; about 10%). American Chemical Society (ACS) guidelines (ACS, 1980) use the term *limit of detection* (LOD) which is the concentration of the analyte at which:

$$\text{Mean sample signal} \geqslant \text{mean blank signal} + 3\ \text{SD} \quad (2)$$

The use of 3 SD (SD = standard deviation) is somewhat empirical and depends on the degree of confidence needed in the assay. Table 1 provides comparative LODs for ELISAs measuring the same analyte, but using different detection systems.

The significance of either Eq. (1) or Eq. (2) is that sensitivity is heavily influenced by NSB. Hence, the mere amplification of signal does not increase sensitivity unless the signal is preferentially amplified over noise. Therefore, reducing NSB is a major consideration in designing highly sensitive ELISAs. Reducing NSB in an ELISA requires identifying its source. Figure 2 illustrates the major sources of NSB in an ELISA (or SPI), which can be collectively grouped into two general categories: (1) lack of reagent specificity of the primary (analyte) interaction (see Fig. 2, arrow a), and (2) immunochemical short-circuitry involving other reactants involved in the pathway leading to the detection system (see Fig. 2; arrows b–g). Within the chain of reactants that characterize most ELISAs, and relative to category a, the immediate distal reactant should recognize *only* the reactant added during the previous stage (see Fig. 2). When the analyte antibody also recognizes determinants other than the desired determinant (i.e., cross-reacts as in a, see Fig. 2), specificity decreases and background increases.

Figure 2 also provides examples of short circuitry involving nonanalyte interactions. For example, if the antiglobulin (anti-IgG1; see Fig. 2) can cross-react with some other bound, non-IgG1 antibody (see arrow d, Fig. 2), specificity will be compromised and NSB increased. If anti-IgG1 itself binds to the solid-phase capture antigen (see arrows c and b), or the antibody–enzyme conjugate binds the solid-phase antigen (see arrows e and g), background is also increased. Other examples of short circuitry are also diagrammed in Fig. 2. In special circumstances, NSB can also result from the presence of endogenous enzyme (see Sec. III). Reagent specificity is a universal

Table 1 The Comparative Sensitivity of Various ELISA-Based Immunoassays for Detecting Thyroid-Stimulating Hormone (TSH)

Method of assay	Analyte	LOD (mol/L)
EIA (fluorescence)[a]	TSH	1.5×10^{-11}
EIA (absorbance)[b]	TSH	2.5×10^{-13}
EIA (fluorescence)[c]	TSH	2.5×10^{-13}
TRF (DELFIA)[d]	TSH	1.0×10^{-13}
CL (acridinium)[e]	TSH	1.4×10^{-13}
CL (enhanced luminol)[f]	TSH	2.0×10^{-13}
CL (acridinium)	TSH	5.0×10^{-14}
CL (dioxentane)[g]	TSH	3.45×10^{-14}

[a]Using β-galactosidase label, microparticle capture, and vacuum filtration.
[b]Using HRP as label and OPD as substrate
[c]Abbott Laboratories TSH label claim, using AP label and the microparticle capture method and apparatus.
[d]Using Amerlite system.
[e]Using magnetizable microparticles separation.
[f]Using coated tubes, no reported criterion for LOD determination.
[g]Using AP-catalyzed dioxetalle and components from commercial EIA kit, 20 min incubation time.
Source: Kahil (1991).

problem in immunology, whereas short circuitry is characteristic of multistage, amplified immunological reactions; the greater the number of components, the greater the opportunity for short circuitry. Complexity generally parallels NSB.

The conventional means of reducing noise is through the use of so-called blocking agents. These are typically nonionic detergents, irrelevant proteins, or a combination of these two ingredients. Detergents are believed to bind primarily to hydrophobic portions of proteins and, thereby, block their hydrophobic interactions with the solid phase. Protein blockers probably act by binding hydrophilically or hydrophobically to other proteins on the solid phase or to the solid phase itself at places where components of the detection system might bind nonspecifically. In some situations, NSB can be reduced by shortening the reaction time, because NSB may be more dependent on secondary bonds (see later) that require more time to form.

Specificity and *sensitivity* are often interrelated concepts; using examples in Fig. 2, sensitivity is lost owing to the lack of absolute reagent specificity. *Absolute specificity* is a feature of the immunoassay, not an inherent feature of antibodies or the immune system. Rather, the designer manipulates concentrations (dilutions), decides on cutoff or confidence levels, selects certain reagents, and optimizes incubation times and agitation levels, to produce the absolute or desired specificity *of the assay*. In other words, specificity is operationally defined. The sensitivity of an assay can thus be improved through reduction in NSB and through changes in assay design (see Sec. IV).

One empirical feature of all solid-phase immunoassay conducted on hydrophobic solid phases depends on a generally accepted tenet; namely that adsorption of the solid-phase reactant is done in the absence of detergents or detergentlike compounds that could inhibit immobilization. [The exception to this principle are those immobilization procedures that are covalent or immunochemical and that do not depend on hydrophobic adsorption. Immobilization of proteins

in detergent cell lysates are an example (see Sec. II.D).] However, in all subsequent steps in ELISAs or SPIs, detergents or other blocking agents are incorporated into the buffers to prevent proteins in subsequent steps from adsorbing nonspecifically to the solid phase. Reactions that are not inhibited by such blockers are assumed to be *specific interactions*.

II. SOLID-PHASE IMMUNOASSAY

A. Immunochemistry of Solid-Phase Interactions

The basic principles of antigen–antibody interactions have been addressed in a number of previous chapters. It suffices here to briefly discuss those unique or particularly characteristic of SPI.

1. Time Required

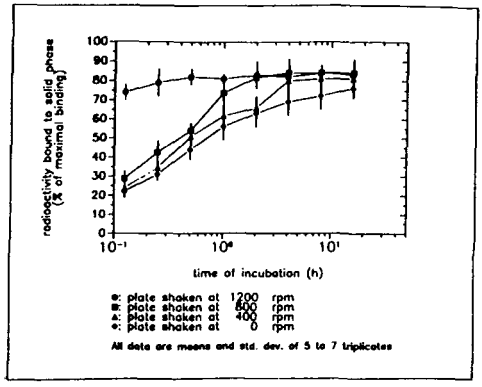
The time required for equilibrium to be reached in SPI is greater than for solution-phase interactions and increases in proportion to the ratio of the volume occupied by the liquid to that occupied by the interfacial receptor. Interfacial reaction kinetics display a pronounced diffusion dependence when conducted in microtiter wells (Fig. 3a). This can be reduced by vortex agitation, which compresses the liquid to a small area in contact with the receptor (reactant)-coated interface (Nygren et al., 1987; Muchens and Scott, 1990; Franz and Stegemann, 1991; see Fig. 3b). This can also be accomplished by forcing the reactive solution phase into a small volume with an inert plunger, by using a porous matrix with a large surface, such as nitrocellulose, or by using microparticles as the solid phase. Microparticles are <1 μm and behave as a colloidal suspension during the assay whereas; particles or beads are larger and settle out by gravity during nonagitated assays; see Fig. 3c. All of these examples illustrate how reducing the ratio of the solution-phase volume to the volume of the reactive interface can reduce the diffusion dependence of SPI.

2. Reactive Volume

The true reaction volume is difficult to determine, but may be much less than the total fluid volume of the reaction vessel. Solid-phase antigen–antibody reactions are confined to the fluid–solid-phase interface, probably to within the attraction distance of the primary bond, which is probably <100 Å. Diffusion or mass transfer (see foregoing) is needed to move reactants into this interfacial volume. The reactive surface area of microparticles (see Fig. 3c) is much larger than for microtiter wells (see Fig. 3b) and constitutes a correspondingly higher proportion of the total volume. The true reactant concentration depends on the true reaction volume, which cannot be precisely calculated. This complicates the calculation of K_{eq} using common graphic forms of the mass laws (see Chap. 30); thus values reported for solid-phase antigen–antibody reactions may be incomparable with those obtained for fluid-phase reactions (Azimzadeh et al., 1991, see Chapter 30).

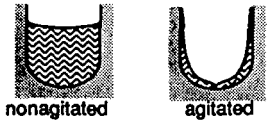
3. Dissociation Rates

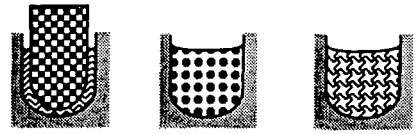
Dissociation rates for interfacial reactions, including those on cell surfaces, may be two orders of magnitude lower than those occurring in solution. The similarity between the slow dissociation rates observed for SPI and for those occurring on cell surfaces, are interesting for several reasons. First, many interactions on cell surfaces involve aggregation of the cell surface receptor, with a corresponding increase in avidity owing to reduced dissociation of multivalent complexes (Metzger, 1992). Not surprising, we and others have shown that at least passively adsorbed antigens and antibodies also appear to be clustered or aggregated (Butler et al., 1992b; Feng et al., 1989; Nygren, 1988; Fig. 4); the active interfacial reactant may be "the cluster." Second, the greater energy needed for dissociation of most antigen–antibody bonds, compared with that



(a)

(b)





(c)

Figure 3 The diffusion-dependence of solid-phase immunoassay and methods used to reduce its influence: (a) The effect of vortexing (shaking) microtiter wells on establishment of equilibrium. (b) Illustration of the physical effect of vortexing microtiter wells (rotary agitation) on the distribution of the fluid-phase relative to the solid-phase. The fluid-phase is depicted by wavy lines. (c) Alternative methods of confining the reaction volume to within close proximity to the solid-phase, which displays the immobilized reactant. [(a) from Franz and Stegeman, 1991.]

needed to prevent their association (hysteresis), indicates that secondary bonds have formed after formation of the initial bond (van Oss et al., 1979; Absolom and van Oss, 1986; see Chapter 23). This suggests that, in SPI and other cell surface reactions, considerable secondary bond formation occurs. Third, a very high solid-phase reactant concentration, especially as might occur in clusters and resulting from a confined interfacial reaction volume (see foregoing), could facilitate more rapid reassociation of dissociated analyte than would occur in fluid-phase systems. This might account for the higher K_{eq} of antibodies when tested in SPI versus in solution (Lehtonen, 1981). Fortunately it is this very slow off-rate that allows ELISA and SPI technology to be so forgiving and for the primary receptor–ligand interaction to remain associated through repeated washing steps.

4. Microtiter Specific Antibody Immunoassay

Microtiter specific antibody immunoassay (SpAbI) using adsorbed complex antigens, may display apparent affinity dependence when compared with their solution-phase counterparts. The stability of solid-phase receptor–ligand interactions seems contradictory to the affinity dependence of SpAbI in microtiter wells (Butler et al., 1978a) and the very low proportion of total antibody captured (Fig. 5). Perhaps this could be due to (1) a very low functional antigen concentration, resulting from a loss of epitopes on adsorbed antigens because of denaturation or steric hindrance; or (2) alteration of epitopes by adsorption such that they are recognized with lower affinity by their paratopes. The stable adsorption of 60–80% of most protein antigens as at least a monolayer (Cantarero et al., 1980; Essar, 1988) when passively adsorbed in the range of 1–5 μg/ml, should not result in a deficiency of total antigen. If 500–800 ng of antigen is stably adsorbed on a microtiter well, a 1:10,000 dilution of serum containing 500 μg antibody per milliliter, should still provide greater than a tenfold excess of antigen. Considering a reasonable K_{eq} for the interaction, a limitation in total adsorbed antigen does not seem to explain the results. Rather, loss of epitopes, either by steric hindrance or adsorption-induced denaturation, seems to be more plausible.

5. Immobilized Reactants

Immobilized reactants, whether antibody or antigen, may not be conformationally displayed in the same manner as in solution. Conformational changes, especially those resulting from their passive adsorption, as well as from other means of immobilization, are well recognized. This is discussed in Sec. II. D–F and reviewed elsewhere (Butler, 1991).

B. An Equation for Solid-Phase Immunoassay

The various immunochemical phenomena observed for SPIs and reviewed in the foregoing, are the basis of Eq. (3)

$$Ag_{SLD^*} + Ab_{SOL} \underset{k_2D}{\overset{K_1D}{\rightleftharpoons}} Ag_{SLD} \rightleftharpoons Ab_{SOL} \underset{k_2R}{\overset{K_1R}{\rightleftharpoons}} Ag_{SLD}\text{–}Ab_{SOL} \underset{k_2A}{\overset{K_1A}{\rightleftharpoons}} (Ag_{SLD}Ab_{SOL}) \qquad (3)$$

Equation (3) describes an interfacial SpAbI using an immobilized antigen (Ag_{SLD}). The equilibrium reaction D governs the diffusion-dependent phase of the reaction whereas R, governed by rate constants k_1R and k_2R, describes the interaction of Ag_{SLD} and Ab_{SOL}, once they have reached the *true interfacial reaction volume*. Whereas the average forward rate constants for solution-phase interaction is $10^7 M^{-1} s^{-1}$ (Karush, 1970; Steward, 1977; Absolom and van Oss, 1986), those for reactions on synthetic solid phase and cell surfaces are two to four orders of magnitude slower (Li, 1985; Nygren et al., 1987). Equation 3 assumes that this slower k_1 is not the consequence of the kinetics of the interaction *within the true interfacial reaction volume*, but the result of slower diffusion and lower mass transfer of reactants to this site. Hence, Eq. 3 distinguishes between D and R and as described above, k_1D can be greatly increased by a number of means (see Fig. 3b,c). Note, the use of k_1 or k_2 without designation such as k_1D, k_1R, k_2A, etc. refers to the overall rate constants described in the literature, not the specific rate constants of Eq. (3).

It is also known that the overall dissociation rate (k_2), from synthetic or cellular interfaces is in the order of 10^{-4}–10^{-5} s^{-1} (i.e., up to two orders of magnitude slower than for solution-phase system (Mason and Williams, 1980; Stenberg and Nygren, 1982; Nygren et al., 1985). Polyvalent interactions in solution also have a higher K_{eq}, which is believed, or has been shown, to result from a lower k_2 (Karush, 1970; Crothers and Metzger, 1972; Azimzadeh and van Regenmortel, 1991). This hysteresis effect, combined with the apparent clustering of solid-phase reactants (see

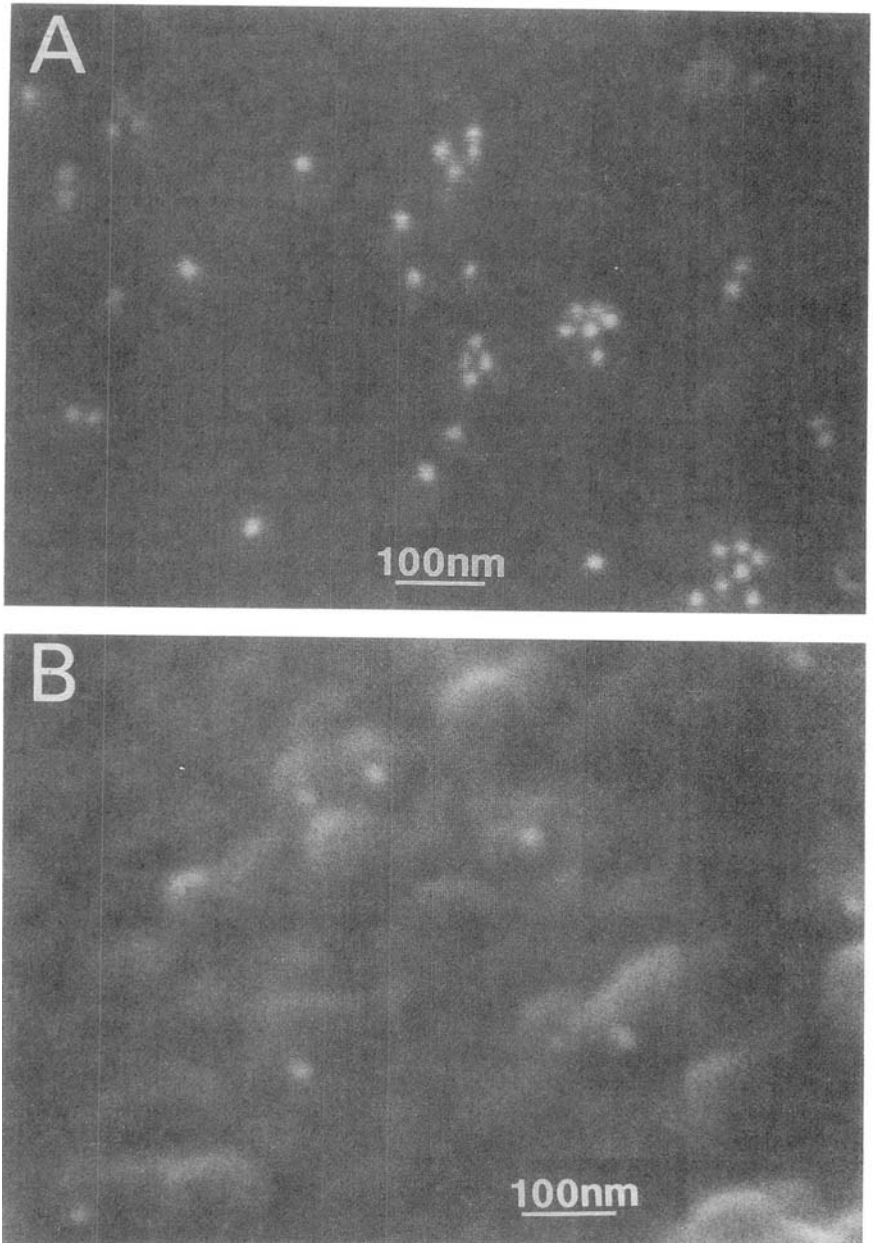


Figure 4 The clustering of surface reactants in ELISA and SPI: (A) The distribution of 20-nm gold-labeled goat anti-IgG after reaction with an Immulon 2 microtiter well to which mouse monoclonal antibodies had been adsorbed at 5μ/ml. (B) The distribution of carbon-coated protein aggregates in microtiter wells during the detection of adsorbed FLU_{10}-IgA by rabbit anti-FLU and goat anti-rabbit conjugated to gold particles. The latter are occasionally visible on the clustered proteins. Dimensions are on the figures.

Fig. 4), suggests multivalent interactions involving secondary bonding to other proteins or to the solid phase itself. Extensive cross-linking at the interface may also be associated with surface coagulation or aggregation by translational diffusion (Michaeli et al., 1980). This could also increase surface contact and further increase hysteresis and lower k_2. Equation (3) treats this secondary aggregation phase A as a third distinct interaction governed by k_1A and k_2A.

Equation (3) is hypothetical, designed to explain observations made by ourselves and others, but, moreover, as a simplistic model for students while challenging them with a testable hypothesis.

C. Types of Solid-Phases and Reactant Immobilization Methods

The aim of in vitro immunoassay is the measurement of the interaction between antigens and antibodies, with the belief that the same interactions occur in vivo. Achieving this aim requires that the immobilized reactant be presented in a configuration representative of that which occurs in vivo.

ELISAs and SPIs are not restricted to plastic microtiter wells, but are performed on a variety of surfaces (see Fig. 3c; Table 2). Differences in volume/surface ratios affect both kinetics and dynamic range (see Sec. II.A), and various chemistries are used in immobilization. Table 2 summarizes the characteristics of the more commonly used solid phases relative to parameters that influence their performance in ELISAs. Solid phases can be grouped into at least three categories, with plastic laboratory ware, such as tubes, microtiter or tissue culture plates,

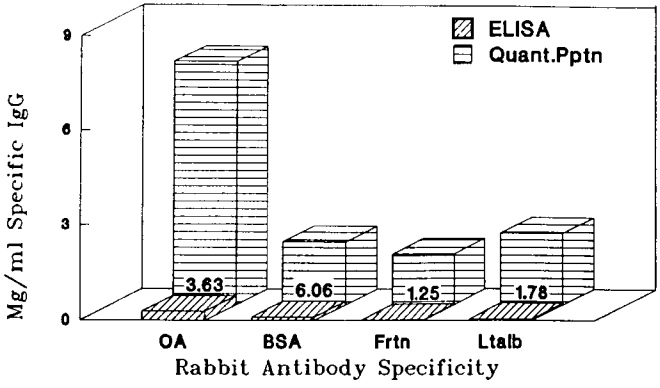


Figure 5 Effectiveness of ELISA in detection of total antibody: The detectability of antibodies to four different protein antigens (OA, ovalbumin; BSA, bovine serum albumin; Frtn, horse ferritin; Ltabl, α-lactalbumin) after the proteins were previously adsorbed on polystyrene, compared with the amount of antibody in the system (Quant. Pptn). Values on the blocks represent the percentage of antibodies detectable by the ELISA-based specific antibody immunoassay (SpAbI).

Table 2 Characteristics of the More Commonly Used Solid Phases That Influence Their Performance in ELISAs

Solid phase	Bonding force	Relative surface area	Performance characteristics
Plastic labware			
Polystyrene	Hydrophobic	Modest	Low background, reproducible, and readily adapted to automation
Polystyrene-irradiated	Hydrophobic and covalent	Modest	
Surface-functionalized polystyrene	Hydrophobic and covalent	Modest	
Bead			
Polystyrene (PS) beads	Hydrophobic	Moderate	Yield assays with broad dynamic ranges; less convenient to use than labware; more difficult to automate
Derivatized PS beads	Covalent, hydrophobic and hydrophilic	High	
Beaded agarose and derivatives	Hydrophilic and covalent	High	Minimal protein denaturation; high background and difficult to automate
Microparticles	Hydrophobic and covalent	Very high	"Solution–phase performance" owing to colloidal nature; wide dynamic range; magnetized variants make them automatable
Membrane			
Nitrocellulose (NC)	Hydrophobic and hydrophilic	Very high	Desorption and background problems hinder their use in quantitative assays
Nylon	Hydrophobic	Very high	Serious background problems reduce signal/noise ratio
Charge-modified nylon	Hydrophilic, covalent, and hydrophobic	Very high	Problems similar to nylon, but perhaps less denaturation and less desorption
Functionalized nitrocellulose	Hydrophobic, covalent, and hydrophilic	Very high	Similar to NC but less desorption
PVDF (Immobilon P)	Hydrophobic	Very high	Very high and stable binding; may be best for immunoblotting

constituting a rather physically and chemically homogeneous group. Not surprisingly, therefore, these materials display similar performance regardless of manufacturer or type. However, subtle differences can affect such things as background, preferential adsorption of certain molecules, and well-to-well variation, but these differences are minor in relation to differences seen among other categories of solid phases (see Table 1). The 96-well microtiter plate is ubiquitous in laboratories in which ELISAs are performed, and at least four companies manufacture plate readers, automatic washers, and diluters designed for such plates; full-robotic systems are also available. The most commonly used material for microtiter plates is polystyrene, with formulations differing among manufacturers. At least two companies, Dynatech and NUNC, offer radiation-treated polystyrene plates that have a somewhat greater capacity to adsorb protein and may also involve covalent bonds owing to the free radicals produced during the irradiation. Both Costar (Cobind) and NUNC (Covalink) offer functionalized polystyrene and, thus, covalent attachment. However, the overwhelming influence of the surrounding hydrophobic polystyrene surface in such wells results in the final protein–plastic bonding being essentially hydrophobic; therefore, performance is only slightly different than on nonfunctionalized surfaces. Functionalized plates may differ substantially in performance from nonfunctionalized plates when used with small molecules or in special applications.

Polystyrene beads function much the same as plates, although providing greater surface area. Their relative inconvenience in use has limited their popularity, whereas microparticle assays have become popular. Microparticles differ from beads in behaving as a colloid, thereby greatly increasing surface area and greatly reducing the diffusion-dependent phase of assays [see Eq. (3)]. The introduction of iron nuclei in some microparticles readily facilitates their separation from the fluid-phase after the reaction. Magnetic beads are also popular in cell separations, in affinity purification of proteins, and in preparation of mRNAs.

Beaded materials composed of carbohydrates are unique on the list in both chemistry and performance. Binding to their hydrophilic surface is usually covalent and proteins immobilized in this manner are only minimally altered (see later). However, hydrophilic beads do not lend themselves to convenient use or to automation and have been largely restricted to affinity chromatography.

The third group of solid phases are membranous and form the basis of ELISA-based immunoblotting (Western blotting; see Chap. 35). Their adsorptive surface area is 100–1000 times greater than plastic, presumably owing to their immense internal surfaces. Also, the porous nature of membranes allow flowthrough technology to be employed in immunoassay, which also reduces the diffusion-dependent phase of the solid-phase immunoassay [see Eq. (3)]. A variety of types exist, including those composed of cellulose nitrate ester (nitrocellulose; NC), nylon, and polyvinylidene difluoride (PVDF). The NC and PVDF are preferred for proteins, whereas nylon is popular for nucleic acids. Membranes, especially nylon, have a high propensity toward NSB binding of proteins, in part because of their large surface area. Thus, they require blocking agents and extensive washing when used in immunoassays. Millipore offers NC in a 96-well format (the well bottoms are NC) and several companies offer assemblies for clamping NC sheets into templates for 96-well systems. The major bonding force to nonmodified membranes is hydrophobic, but there is also evidence for some hydrophilic bonding. It is possible that a certain amount of the latter represents protein–protein bonds that dissociate under conditions designed to break hydrophilic bonds (Brown et al., 1991).

Solid-phase reactants can be immobilized by three general procedures: (1) passive adsorption, resulting from strong hydrophobic interactions between proteins and the synthetic surface; (2) covalent attachment to functionalized solid-phases; and (3) immunochemical and other nonadsorbent, noncovalent attachment. The latter includes immobilization by streptavidin–biotin linkages, the use of capture antibodies, or the use of bacterial immunoglobulin-binding proteins

(e.g., protein-A). Covalent attachment is most common for hydrophilic beads (agarose) or heavily functionalized polystyrene beads. Passive adsorption is most widely used for ELISAs on microtiter plates and for immunoblotting on NC (see Chap. 32).

D. The Immobilization of Proteins by Passive Adsorption

The adsorption of proteins on synthetic surfaces, such as polystyrene latex and glass, was studied as early as 1956 (Bull, 1956), although its popular application to immunoassay dates to Catt and Tregear (1967). Figure 6 illustrates the characteristics of adsorption at alkaline pH on polystyrene. Passive adsorption under these conditions follows typical saturation principles. Viewed as percentage bound plots, the avidity differences among protein for the plastic are large and appear correlated with molecular mass (see Fig. 6). Note the term *affinity*, as applied to SPI, is probably more correctly *avidity*, as it reflects a multivalent, aggregation-type interaction. Calculations indicate that saturation occurs when a monolayer of protein has become adsorbed (Cantarero et al., 1980; Butler et al., 1992b; Esser, 1988), which is consistent with data showing that fewer large molecules are needed than small molecules (see Fig. 6). Saturation with IgG antibodies corresponds to the addition of about 1000 ng/200 μl in a microtiter well (Cantarero et al., 1980; Sorensen and Brodbeck, 1986). Most capture antibodies also have their optimal performance when adsorbed at this concentration (Joshi et al., 1992).

Nitrocellulose and other membranes show (1) a much higher capacity for adsorption per planar surface area than plastic and (2) considerable adsorption heterogeneity among membranes and proteins (Brown et al., 1991). In contrast, adsorption of IgG on polystyrene microtiter wells from various manufacturers at alkaline pH, differs only subtly (Butler et al., 1991).

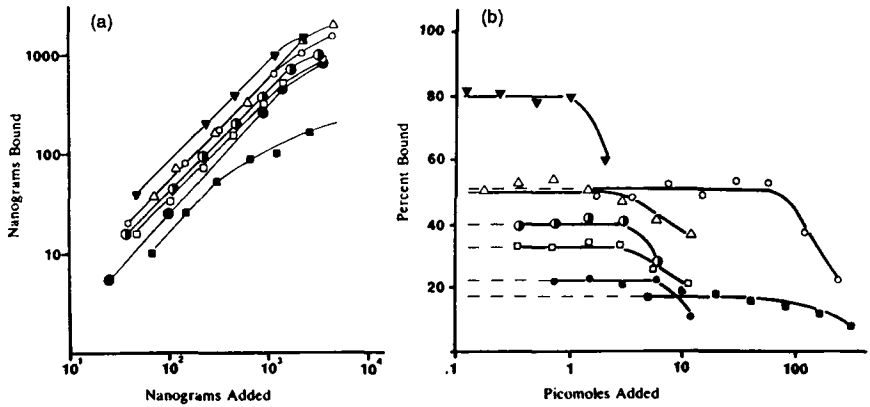


Figure 6 The characteristics of passive adsorption on polystyrene at pH 9.6: (a) The adsorptive behavior of seven different proteins in a log–log saturation plot. ▼, bovine IgM, 1000 kDa; △, bovine SIgA, 420 kDa; ○, ovalbumin, 44 kDa; ◑, bovine IgG1, 158 kDa; □, bovine IgG2a, 152 kDa; ●, bovine serum albumin, 69 kDa; ■, bovine α-lactalbumin, 14 kDa. (b) the adsorptive behavior of the same proteins expressed in a molar percentage bound plot. Dashed lines are extrapolations in the region of constant percentage binding for which empirical data were not reported. Same symbols as in (a). (From Cantarero et al., 1980.)

The consequence of passive adsorption of proteins on polystyrene has been studied by various investigators who generally reach the same conclusions (see reviews Butler, 1991; Butler et al., 1992). Namely, passive adsorption results in the loss or alteration of antigenic epitopes, the loss of enzymic activity, the generation of new epitopes, demonstrable physicochemical changes, loss of CAb activity (Suter and Butler, 1986; Butler et al., 1992b) or reduction in CAb affinity (Arends, 1971). Losses in antibody activity can exceed 90%, and if the same occurs with antigen, it can explain the discrepancy between total serum antibody measured by quantitative precipitation and that measured by SpAbI ELISA (see Fig. 5). At least for polystyrene, there appears to be sufficient evidence that passive adsorption involves conformational changes as proteins unfold to permit internal hydrophobic side chains to form strong hydrophobic bonds with the solid phase. Protein adsorbed on polystyrene is very stable if adsorption occurs within the linear-binding region (see Fig. 6); repeated washing with detergent and protein-containing solutions will not dislodge these protein.

The biological consequences of adsorption on various blotting membranes appear to have been of less concern than adsorption on polystyrene. Even if denaturation is on par with that which occurs on polystyrene, much more protein binds to NC, so that enough native protein may survive to permit denaturation to be overlooked. Furthermore, evidence that hydrophilic as well as hydrophobic forces are involved in the adsorption on immunoblotting membranes suggests that conformational alterations may not be as severe as they are on polystyrene.

If passive adsorption is so destructive for proteins, why is there continued use of this procedure? The answer is simply that enough molecules survive to make the assay work. For example, if 6% of high-affinity capture antibodies adsorbed on a microtiter well survive in a functional state, it would be sufficient to provide a sandwich ELISA (Fig 1b) with a dynamic range (2–200 ng/ml); this 2 log range is adequate for most applications and typical of assays reported in the literature.

E. Immobilization of Other Macromolecules and Peptides

Polysaccharides and heavily glycosylated proteins often have low affinity for polystyrene (Barret et al., 1980; Carlson et al., 1982; Katz and Schiffman, 1985; Voller et al., 1976; Zigterman et al., 1988) and often require alternative methods for their immobilization. One alternative is covalent immobilization using a suitable cross-linking agent such as glutaraldehyde, EDAC, or dimethyl suberimidate, to (1) functionalized polystyrenes, such as commercially available aminostyrene (e.g., Covalink from NUNC, Roskilde, Denmark); (2) polystyrene first treated with surface-modifying agents to produce isocyanate groups (Saito and Nagai, 1983) or amino groups (Chin and Lanks, 1977; Quash et al., 1989; Rubin et al., 1980; Thomas et al., 1990); or (3) polylysine, phenylalanine-lysine, octadecylamine, or some irrelevant protein that is adsorbed beforehand (Hobbs, 1989; Parsens, 1981; Papadea et al., 1985; Suter, 1980). The same method has been employed for the immobilization of proteins that (1) because of their chemistry cannot bind to a hydrophobic surface, (2) are so small that their affinity may be very low (see Fig. 6), or (3) are conformationally altered by adsorption to the extent that they are nonfunctional (Shirahama and Suzawa, 1985; Lauritzen et al., 1990; Sødergard-Anderson et al., 1990). On the other hand, others have successfully adsorbed bacterial capsular polysaccharides on plastic (Grantstrom et al., 1988), and a surprising number of investigators have merely adsorbed peptides passively to polystyrene (Atassi and Atassi, 1986; Geerligs et al., 1988; Lacroix et al., 1991; Neurath et al., 1990), or synthesized them directly on polystyrene rods (Geysen et al., 1984). Unfortunately, there are few studies that address the antigenic consequences of passive peptide adsorption. Sødergard-

Anderson et al. (1990) observed that angiotensins I and II were five- to tenfold more antigenically active when covalently attached than when adsorbed. Lacroix et al. (1991) observed that the form of the peptide was important; cyclized peptides appeared more active than their linear counterparts.

Another group of biomolecules often studied are those freed from membranes by cell lysis in the presence of detergents. Because detergents are used to block adsorption to hydrophobic surfaces, their presence will inhibit adsorption of the lysate proteins to plastic (Gardas and Lewartowska, 1988; Kenny and Dunsmoor, 1983; Newman et al., 1981). Adsorption to NC is possible, particularly if detergent concentrations are lower than 0.01%. Triton, Tween, and sodium dodecyl sulfate (SDS) are most inhibitory, whereas deoxycholate and octylglucoside have the least effect (Palfree and Elliott, 1982). That detergents are much more inhibitory to adsorption on nylon (Grenescreen) than on NC, supports the idea that forces other than hydrophobic may be involved in adsorption to NC (see Table 2).

When detergent levels in cell lysates cannot be diluted to permit adsorption, covalent linkage may be used (Newman et al., 1981; Evans, 1984) or materials such as SM-2 Bio-Beads can be used to reduce the detergent concentration (Drexler et al., 1986; Van Kreveld and Van den Hoed, 1973).

Two other alternative methods for immobilizing biomolecules that do not lend themselves to passive adsorption are immunochemical immobilization and other noncovalent methods of immobilization.

Immunochemical immobilization typically involves the use of an adsorbed capture antibody to immobilize the antigen or antibody of interest in SpAbI (Hirano et al., 1989; Nustad et al., 1984; Zeiss et al., 1973). The inherent problem with this approach is that only 10–25% of the capture antibody may survive denaturation on polystyrene, so that as few as one-in-ten capture antibody molecules are functionally able to capture antigen. This reduces the concentration of antigen much below the total antigen concentration that can be achieved by simple adsorption. Nevertheless, immunochemically immobilized antigen can be tenfold more active than adsorbed antigen (Herrmann et al., 1979) which, combined with the much lower amount present, further supports the idea that a high proportion of adsorbed antigen is antigenically inactive (see foregoing). A major application of immunochemical immobilization is the immobilization of proteins in detergent-containing cell lysates and in samples containing complicating contaminants (see virus immobilization in the following section).

The high affinity of avidin and streptavidin (SA) for biotin (Green, 1963; Prian and Riordan, 1990) provides an alternative, nonadsorptive, noncovalent means of immobilizing both antigens and antibodies. Because of the low affinity of SA for polystyrene, it must be (1) first covalently bound to the surface (Peterman et al., 1988; Nardelli et al., 1989), (2) immobilized by an irrelevant, biotinylated carrier (Suter and Butler, 1986), or (3) immobilized by biotin covalently attached to functionalized polystyrene (Bugari et al., 1990). Each of these methods permit the immobilization of antigens and antibodies that bind poorly to plastic or NC and are denatured beyond use by adsorption.

Lectins and the immunoglobulin-binding proteins of bacteria, which are readily adsorbed on plastic or other hydrophobic surfaces, can also be employed as a bridge between the solid phase and the reactant of interest. Prerequisite is that the adsorption process does not destroy or alter their specificity. Concanavalin A (ConA) adsorbed to microtiter wells is able to immobilize gp120 of the human immunodeficiency virus (HIV), which displays better activity than adsorbed gp120 (Robinson et al., 1990). Both protein A of *Staphylococcus aureus* and protein G of *Streptococcus* spp. are capable of stably capturing various IgGs after their adsorption. The potential background problems connected with the use of IgG-binding proteins in ELISAs are discussed in Secs. IV and V.

F. Cells, Bacteria, and Viruses as Solid-Phase Reactants

Virus particles can be directly adsorbed on polystyrene in the manner of proteins (Butler, J.E., unpublished; Herrmann et al., 1979; McCullough et al., 1985). Our experiences reveal two potential problems with this approach. First, the virus must be purified free of tissue culture protein, which can competitively inhibit its adsorption. Second, virus preparations bound on the solid-phase must be free of any antigens (normally of media origin) that may have been present in the vaccine preparation, since the sera of immunized animals or vaccinated humans, may also contain antibodies to these proteins. This problem has been overcome by using an adsorbed, virus-specific capture antibody to immobilize the virus (Herrmann et al., 1979; McCullough et al., 1985; Trent et al., 1976; Yolken et al., 1980). Although predictably fewer virus can be immobilized by this method than by adsorption (Herrmann et al., 1979), exposure of internal epitopes caused by adsorption-induced denaturation is reduced (McCullough et al., 1985). Adsorbed CAbs have also been used to detect the presence of virus (Voller et al., 1976; Miranda et al., 1977).

Intact cells and bacteria have also been used as solid-phase ELISA antigens. Their heavily glycosylated membranes usually hinder their stable adsorption; consequently, simultaneously adsorbing and cross-linking them to surfaces coated with poly-L-lysine (Franci et al., 1988; Mazia et al., 1975; Suter et al., 1980), glutaraldehyde (Severson, C., and Bishop, G. A., personal communication, 1985, 1990) or the polyaldehyde methyl glycosal (Czerkinsky et al., 1983) have been used. This issue will be further discussed in Sec. IV.D.

III. ENZYME-MEDIATED REPORTER SYSTEMS

A. Popular Enzymatic Systems

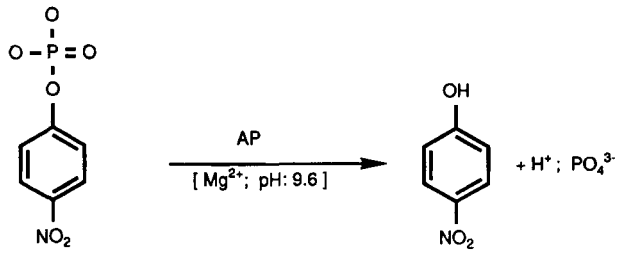
At least 15 different enzymes have been used as signal generators for HetEIA, although three, horseradish peroxidase (HRP), alkaline phosphatase (AP), and β-galactosidase (βgal), are used most widely (Ngo, 1991; Table 3). All three enzymes possess desirable qualities: availability of sensitive substrates, adaptability of substrate systems to further amplification, enzyme stability, ease of conjugation to antibodies and other proteins, manageable environmental interference, availability, and an established "track record" in science. Commercial suppliers of enzyme-conjugated antibodies appear to favor HRP and AP. β-Galactosidase is less widely used, but is popular in some kit assays, in some ultrasensitive ELISAs (Ishikawa and Kato, 1978), and for cell ELISAs (see later). All three are stable and catalyze highly sensitive reactions, although each has its disadvantages; examples include the disagreeable odor of mercaptans in some substrates for βgal, the expense of AP, the rapid kinetics of HRP (also an advantage in some cases), and the interference by endogenous peroxidases and phosphatases in some specimens, especially in cell ELISAs. Another factor can be molecular size (see Table 3); the immunochemical consequences of steric hindrance in ELISA (see Sec. IV and V) favors the use of the smaller HRP (Koertge and Butler, 1985; Butler et al., 1986).

Alkaline phosphatase is a mammalian enzyme available in various forms and is often the most expensive of the three most commonly used (see Table 3). *p*-Nitrophenyl phosphate (pNPP) is the most commonly used substrate when a soluble product is desired. It is converted to *p*-nitrophenyl with a λ_{max} of 405 nm. The enzyme reaction is virtually "bullet proof" and a very stable colored product develops more slowly than those generated with HRP. Hence, we have found AP to be more convenient in a research environment. Both the V_{max} and K_m of AP increase with pH to >10. Alkaline phosphatase is a zinc metalloenzyme, the activity of which is enhanced by magnesium, as well as by increased pH. Naturally, chelators such as EDTA are inhibitors, as well as PO_4^{-2}, which inhibits competitively. The actual catalytic reaction is more complex than that

Table 3 Common Enzyme and Enzyme Systems Used in ELISA

Enzyme (abbrev.)	Source	Activity (U/mg)	Approximate cost ($/1000 U)	M_r (kDa)	pH optimum	Colorimetric (abbrev.; max)	Fluorescence (F) chemiluminescence (CL)
Alkaline phosphatase (AP)	Mammal, typically calf intestine	2000	17	84–150	9–10	*p*-Nitrophenyl phosphate (pNPP; 405 nm) Phenolphthalain monophosphate (PPM) 5-Bromo-4-chloro-3-indolyl phosphate (5-BCIP) Nitroblue tetrazolium (NBT) NADP	β-Naphthyl phosphate (F) 4-Methylumbelliferyl phosphate (F) 3-*O*-Methylfluorescein monophosphate (F) Lumigen PPD (CL) AMPPD (CL) CSPD (CL)
β-galactosidase (βGal)	*Escherichia coli*	250–500	20	540	6–8	O-Nitrophenyl-β-D-galactopyranoside (ONGP; 420 nm) 2-Naphthyl-β-D-galactoside Chlorophenolic red-β-D-galactopyranoside (CPGP; 574 nm)	Methylumbelliferyl-β-D-galactoside (F) AMPGD (CL)
Horseradish peroxidase (HRP)	Horseradish	1000 (for ABTS)	3.2	40	5–7	4-Chloro-1-naphthal (4CN) 5-Aminosalicyclic acid (5-ASA) *O*-Phenylenediamine (OPD; 492 nm) 2,2′-Azino-di(3-ethylbenzthiazoline) sulfonic acid (ABTS; 415 nm) *O*-dianisidine (ODIA) 5-Aminoantipyrine (5AS) Dicarboxidine, 3,3′,5,5′-tetramethylbenzidine (TMB; 450 nm) 3-Dimethylaminobenzoic acid (DMAB) 3,3′ Diaminobenzidine (DAB)	Luminol (CL) Lucingenin (CL) 3′,6′-Diacetyl-2′,7′-dichlorofluorescein (F) 3-(4-Hydroxyphenyl)-propionic acid (F) 4-Hydroxyphenylacetic acid (F)

Western Light uses two chemiluminescent substrates for AP, AMPPD (disodium 3-(4-methoxyspirol[1,2-dioxetane-3,2-tricylo[3.3.1.1^{1,7}]decan-4-yl)phenyl phosphate and CSPD (disodium 3-(4-methoxyspiro(1.2-dioxetane-3,2′(5-chloro)tricyclo[3.3.1.1^{1,7}] decan)-4-yl)phenyl phosphate. AMPGD is the "AMPPD equivalent" for use with β-gal.



(4)

pNPP p-nitro phenol

in Eq. (4) and involves various intermediate complexes (Ngo, 1991). Alkaline phosphatase is extremely stable. Stored in glycerol at −20°C, we have observed virtually no reduction in the performance of soluble immune complexes containing AP after storage for 5 years (Koertge et al., 1985).

Horseradish peroxidase made its debut into immunology through the field of immunohistochemistry (Nakane and Pierce, 1966). It is relatively small, can be conjugated to protein by a variety of methods (see later), and a rather wide variety of aromatic phenols can serve as hydrogen donors for the peroxide oxidation that it catalyzes. Of the many indicators (see Table 3), we have used 2,2′-azino-di(3-ethylbenzthiazalone) sulfonic acid (ABTS) quite successfully, when soluble products are acceptable, and bisdiazobenzidine (BDB) or *o*-phenylenediamine (OPD), when insoluble products are needed (see Sec. IV.C, ELISPOT). A variety of the substrates for HRP, including those just mentioned, are carcinogenic. 3,3′,5,5′-Tetramethylbenzidine (TMB) also yields sensitive assays and is neither mutagenic nor carcinogenic (Van Weemen, 1985). Plate readers equipped with an interference filter of 405 nm, can be used to measure both the products of AP acting on pNPP and HRP when ABTS is used (see Table 3). The actual mechanism of HRP catalysis includes oxidation of the enzyme itself to HRP and compound I, which accepts hydrogen from one of the donor substrates to become HRP compound II. Further reduction by the hydrogen donor converts compound II back to free HRP (Ngo, 1991). Care needs to be given to maintenance of pH and to the quality and molarity of H_2O_2 used.

Horseradish peroxidase exists in the form of various isoenzymes; isoenzyme C is the preponderant cationic form and constitutes the bulk of commercially purified HRP (Ngo, 1991). This enzyme is not as stable in soluble form as AP and is inactivated slowly at 4°C in the presence of PO_4^{-2}. Storage is best accomplished in more inert buffers, such as borate or carbonate, with glycerol added to prevent proteolysis and aggregation of protein–HRP conjugates. Tween may also delay inactivation (Porstmann et al., 1981b).

The small size of HRP, the ease with which small conjugates can be produced through oxidation of its carbohydrate moieties to reactive aldehyde (discussed later), its rapid kinetics, and reasonable price, make it popular in ELISA. Reproducible performance probably requires more concern for substrate and H_2O_2 quality and assay conditions than is required for AP. It is less stable than AP, but this is a problem only when conjugates are not rapidly consumed by a laboratory. It shares with AP the potential danger that when used with some eukaryotic tissue, cells, or body fluids, residual peroxidase or phosphatase activity can cause unacceptable backgrounds.

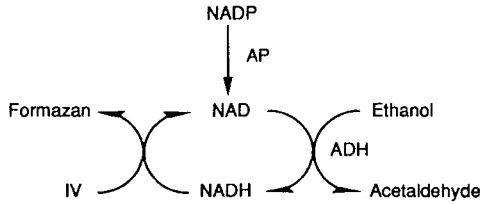
β-Galactosidase is a multimeric enzyme of *Escherichia coli*, the structure and genetics of which have been well studied. The βgal gene encodes a protein of 116 kDa, which forms functional tetramers of about 540 kDa. The βgal substrates are very stable, but catalyze reactions with slower turnover rates than HRP or AP. It is not found in the tissues or body fluids of higher organisms and, therefore, is free from background problems resulting from endogenous phosphatases or peroxidases.

β-Galactosidase catalyzes the hydrolysis of terminal nonreducing β-D-galactose in β-galactosides and is specific for the D-galactopyranoside moiety and β-glycoside linkage. *o*-Nitrophenyl-β-D-galactopyranoside is the common colorimetric substrate, which is dissolved in a phosphate buffer, at neutral pH, containing magnesium and 2-mercaptoethanol.

The use of these three enzymes is not limited to colorimetric assays, but as described in the following, they are also used to generate fluorescent and chemiluminescent signals and in enzyme cascade or cofactor-cycling amplification systems (see Table 3).

B. Fluorescence, Chemiluminescence, and Other Amplification Systems

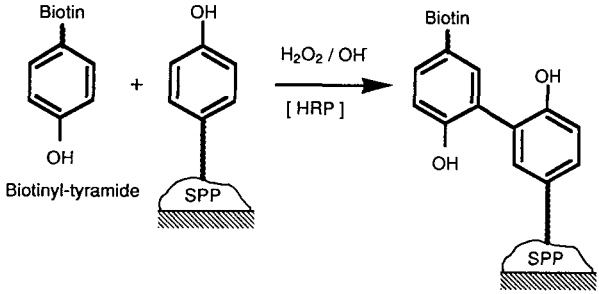
Increasing the sensitivity or LOD of ELISAs [see Eq. (2)] may be achieved immunochemically or through nonimmunochemical methods that amplify the enzyme–substrate–product circuit. An example of the latter is cofactor-recycling amplification, as described by Self (1985) for AP [Eq. (5)]. In this system, the dephosphorylated product (NAD) can initiate a cyclical reaction that



(5)

effectively amplifies the formation of the colored product formazan from the colorless iodonitrotetrazolium violet (IV) which is simultaneously oxidized back to NAD to initiate another cycle.

Catalyzed reporter deposition (CARD; Bobrow et al., 1989), is an example of signal amplification that results from increasing the enzyme/analyte ratio. Catalyzed reporter deposition is based on the principle that HRP can catalyze oxidative condensation through a free-radical mechanism and, in particular, can cause dimerization of phenols [Eq. (6)]. The user selects the



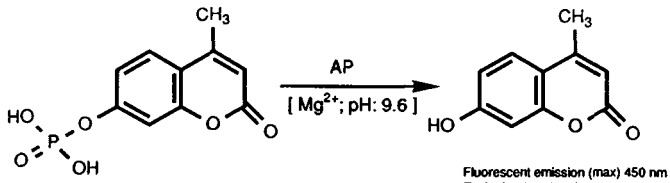
(6)

appropriate heterogeneous ELISA configuration (see Fig. 1) by the criteria discussed on pp. 782 and 788 and performs the assay in the normal manner until the step involving addition of the enzyme–conjugate. At this point, the first phase of the CARD detection system is initiated by the addition of the HRP-labeled detector antibody in the presence of biotinylated tyramide [see Eq. (6)]. The HRP then catalyzes the phenolic dimerization of biotinyl-tyramide to any tyrosine phenol group in the immediate vicinity [e.g., the protein side chain of any available solid-phase protein, SPP; see Eq. (6)]. Confinement of deposition to only phenolic groups in the vicinity

of the initial interaction is believed to result from the very short half-life of the phenolic free radical. The biotinylated phenolic dimer now serves as a receptor for streptavidin–AP, which leads to a higher AP/analyte ratio and greater than 1 log increase in sensitivity (Bobrow et al., 1989). By use of a modified sandwich ELISA and CARD, Helle et al. (1991) reported an LOD of 5×10^{-15} mol/L in the detection of interleukin-6. The system appears especially suitable for lowering the detection limits in immunoblots using BCIP/NBT as the depositional substrate of AP; a greater than 2-log improvement has been reported for such applications (Bobrow et al., 1991). The CARD system is commercially marketed as ELAST by DuPont.

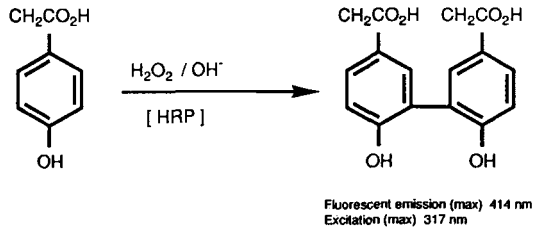
The *amplified ELISA* (a-ELISA) achieves a signal increase by the same principle as CARD (i.e., increasing the enzyme/analyte ratio; Butler et al., 1978b; 1985). Furthermore, in the a-ELISA scheme, AP is not covalently conjugated to the detector antibody, but is provided in the form of a novel, soluble immune complex which, at the alkaline pH of the AP–substrate reaction, results in the dissociation of the noncovalent immune complex so that AP is released to act in free solution with pNPP (see following Sec. III.B). When used as an indirect competitive ELISA, the a-ELISA is dubbed CELIA (Yorde et al., 1976).

The cofactor-recycling system illustrated in Eq. (6) is novel among nonimmunochemical methods of signal amplification; the use of fluorescence or chemiluminescence, which lowers the LOD by increasing the detectability of the signal, is the more common nonimmunochemical means of signal amplification. Fluorogenic substrates are available for the major enzymes used in ELISA (see Table 3) and the structure of those often used with AP [Eq. (7)] and HRP [Eq. (8)] are



(7)

4-methylumbelliferylphosphate 4-methylumbelliferone



(8)

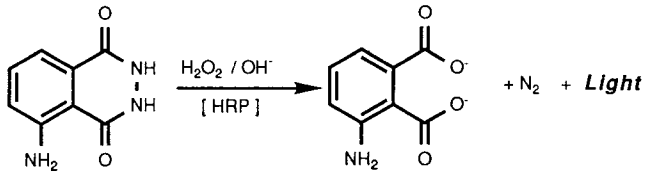
p-hydroxyphenylacetic acid oxidized dimer

presented. Theoretically, fluorimetry is about 2 logs more sensitive than colorimetry (Khahil, 1991) although this advantage can be compromised by NSB. Fluorescence from serum components is problematic; hence, HetEIAs, which permit removal of extraneous protein during wash steps, on the one hand, are more applicable to the use of fluorescence detection system than HomEIA (Kato et al., 1979). On the other hand, light scatter is a greater problem on solid phases than it is in solution. The improvement in sensitivity using fluorescence detection can also be time-dependent, as fluors, similar to radioisotopes, exhibit signal decay; this might explain the

observations by Yolken and Leister (1982) in which the advantage of fluorescence over colorimetry decreased with time.

Successful fluorometry depends on numerous factors, including the excitation source, optical system, assay configuration, and choice of fluor. Fluorescein (FLU), perhaps the best-known fluor, with a quantum yield of over 0.95, has a number of limitations. For one thing, its Stokes shift is small (492–520 nm), so eliminating all excitation radiation from the emission can be difficult, and when this is accomplished using narrow emission filters, considerable energy (signal) is lost. Furthermore and as aforementioned, the emission spectrum of FLU is similar to that of many serum components. Therefore, fluorescent lanthamide chelates have been introduced to eliminate these disadvantages (Table 4). The lanthamide chelate europium (Eu) is the basis of time-resolve fluorescent (TRF) systems such as DELPHIA (Pharmacia, Upsalla, Sweden). In TRF, the fluorescence of the long-lived Eu^{3+} can be discriminated from short-lived endogenous fluorescent light. Use of TRF and fluors, such as Eu^{3+}, also permit higher-energy excitation (e.g., lasers) to be used, which when used with FLU would simply amplify signal and background equally. Recently, a streptadivin-based macromolecular complex of Eu^{3+} has been used in a HetEIA to detect 0.5 attamoles (300,000 molecules) of alpha-fetoprotein (Diamandis, 1991). Although the improved sensitivity of optimized fluorescence is well demonstrated in such systems as DELPHIA, PCFIA*, and the Abbott TDx, its use in microtiter systems has yet to receive widespread attention.

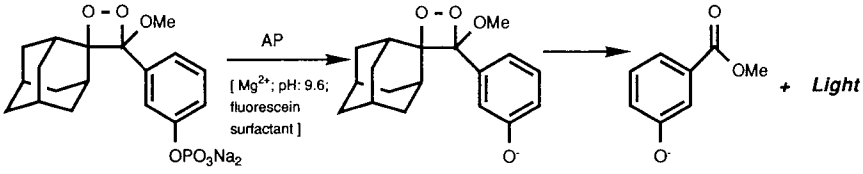
A popular and, to date, the most effective means of signal amplification (see Table 3) is chemiluminescence (CL). Luminescence involves the release of energy as photons when excited atoms or molecules transition to a ground state. *Chemiluminescence* is a subcategory of *luminescence*, in which the excited state is the result of a chemical reaction. For example, HRP at alkaline pH is able to catalyze the conversion of the phthalhydrazide luminol to the dicarboxylate ion, which is considered the light-emitting species [Eq. (9); McCapra, 1974). Another example



(9)

Luminol

of CL that employs HRP as the catalyst, is the conversion of aryl acridinium esters (e.g., lucigenin) at alkaline pH, to *N*-methylacridone, which emits photons at 430 nm [Eq. (10)]. A CL system that we have found useful with AP uses Lumigen PPD (Boehringer-Mannheim) that, when dephosphorylated, yields an unstable intermediate that decays, resulting in light emission [see Eq. (10)]. Additional CL substrates for AP are available from Tropix, Bedford, Massachusetts, some as part of a kit detection system called "Western Light."



Lumigen PPD

(10)

Table 4 Advantages of Lanthamide Chelates in Comparison with Conventional Fluorophores

Characteristic	Conventional fluorophores	Lanthamide chelates[a]
Stokes shift	Small (28 nm for fluorescein)	Large (290–300 nm for Eu^{3+}
Emission band	Wide	Narrow
Lifetime	Short (<100 ns)	Very long (1–100 μs)
Overlap of excitation–emission spectra	Significant	Negligible to nonexistent
Overlap with serum autofluoresence	Significant	Negligible
Pulse excitation[a]	Difficult	Simple
Light scatter	Severe	Nonexistent
Background fluoresence[b]	Significant	Nonexistent
Quantum yield	Excellent	Good
Detection limit	$\sim 10^{-9}$ mol/L	$\sim 10^{-13}$ mol/L

[a]Used with microsecond time-resolved fluorometry.
[b]Optics, solid phases, solvents, impurities.
Source: Diamandis et al. (1991).

The LODs for luminol, the acridium ester and Lumigen PPD systems range from 10^{-12} to 10^{-15} mol/L; the lower values places them in the most sensitive general category of signal amplification systems (see Table 1).

The sensitivity of CL may neither be required nor justify the equipment costs needed for its applications in routine microtiter or microparticle ELISAs. Chemiluminescence may have its greatest impetus in the field of immunoblotting, not just in the detection of antibody–antigen interactions, but as an alternative to ^{32}P in DNA hybridization experiments (see Sec. VI). In contrast to fluorescence and colorimetry, but akin to radioactivity, CL is a detection system that chemically emits radiation that can activate photographic emulsion. Hence, CL can be measured by autoradiography, thereby providing a permanent blot record using automated equipment readily available. Furthermore, chemiluminescent blots may be repeatedly stripped and reprobed, whereas the deposition enzyme products accumulated when colorimetric procedures are used are difficult or impossible to remove. Finally, quantitative CL requires more fine-tuning than colorimetric assays, an issue of little concern in qualitative immunoblots. There are several CL products available for use with both HRP and AP (see Table 3).

Conversion of colorimetric detection to chemiluminescent detection typically involves higher dilution of reactants (reagents) to reduce NSB. In general, laboratories that have taken the time to optimize their assays are generally able to achieve data equivalent to that obtained with radioisotopes (see Sec. VI).

C. Enzyme–Protein Conjugates

The conjugation of enzymes to proteins is as fundamental to the concept of ELISA as the use of enzymes as reporters. Conjugation methods can be grouped into three general categories: (1) procedures involving random covalent cross-linking of enzyme and protein, (2) controlled covalent coupling of specific chemical groups, and (3) complexes coupled through noncovalent bonds.

Each of these procedures has its advantages and disadvantages. Random copolymerization using, for example, glutaraldehyde, results in conjugates of various sizes, many exceeding 1000 kDa, with enzyme activity reduced to 15–60% of that of the free enzyme (Engvall, 1978;

Avrameas, 1969; Gugerli, 1983; Porstmann and Kiessig, 1992). Conjugates of large size can sterically hinder analyte detection, whereas conjugates with low relative molecular mass (M_r) and those composed of a single Fab fragment plus one molecule of AP, show the least steric hindrance (Koertge and Butler, 1985; Butler et al., 1986). However, the latter conjugates display a lower enzyme/analyte ratio (i.e., they do not amplify detection; Koertge and Butler, 1985).

Chemically targeted conjugation is exemplified by the conjugation of HRP (40 kDa) to antibody (150 kDa) by periodate oxidation of the carbohydrate moiety of the antibody (Nakane and Kawaoi, 1974; Wilson and Nakane, 1979). Such conjugates are easy to prepare, are of reasonable size, and HRP may retain 80% of its activity after conjugation. The HRP isoenzymes with alkaline pIs retain the highest activity (Porstmann et al., 1987).

Other types of targeted coupling involves the use of various heterobifunctional cross-linking agents (Pierce Chemical Co., Rockford, Illinois). For example, the amino groups of a protein can first be reacted with the succinimdyl portion of succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), and the maleimide portion of the cross-linked protein then reacted in a controlled manner with the thiol groups of βgal (Ishikawa et al., 1983). The participation of the same cross-linker can be reversed, first complexing the amino groups of AP and then the thiols of antibody Fab fragments.

The use of a soluble enzyme immune complex (EIC) for immunoassay was pioneered by Sternberger et al. (1970) who used peroxidase–antiperoxidase complexes as signal generators in both light and electron immunohistochemistry. A similar approach was proposed by Lenz (1976) and developed by Butler et al. (1978a) as the a-ELISA and by Yorde et al. (1976) for indirect competitive HetEIA (CELIA). Although such conjugates (immune complexes) suffer from the same steric hindrance problems as randomly cross-linked conjugates (Koertge and Butler, 1985), they offer a unique advantage in ELISA systems, because the addition of the substrate results in the dissociation of the EIC, release of free AP, and restoration of activity (Koertge et al., 1985; Butler et al., 1985). Optimal EICs form with a ninefold molar excess of enzyme (Koertge et al., 1985), resulting in a considerable amount of free enzyme in solution. Similar results have been obtained using antienzyme MAb (Ternynck et al., 1983). Another advantage of EICs is that all antibody is enzyme-bound, a situation that complicates covalently prepared conjugates that contain active antibody not bound to enzyme, unless removed beforehand (Porstmann et al., 1981a). Other variants of the EIC approach are the formation of analyte-bound antienzyme (Guesdon et al., 1983) and the use of biospecific antibodies (Porstmann et al., 1984; Karawejew et al., 1988).

The topic of conjugate preparation, using reagents described here as well as others, is reviewed elsewhere (Ishikawa et al., 1988; Ngo, 1991; Porstmann and Kiessig, 1992).

IV. ANTIGEN QUANTITATION

A. Choosing the Assay Configuration

Three assay configurations may be employed for antigen quantitation: (1) competitive (see Fig. 1c, d); (2) sandwich or two-site (see Fig. 1b); and (3) homogeneous enzyme immunoassay (HomEIA). The choice of configuration and the particular variant of that configuration depends on the following criteria:

1. Quantitation versus detection
2. The valency and size of the antigen
3. The sensitivity desired
4. The time constraints on data collection
5. The accuracy desired

Choice of configuration is largely independent of the type of solid-phase used (see Table 2) except HomEIA (which lacks one) and immunoblotting, which involves transfer of antigens to membranes. Often field or laboratory application and commercial availability determine solid-phase selection. Assays span the gauntlet from (1) semiquantitative systems designed to identify antigens separated by electrophoresis, (2) assays designed for positive or negative selection, (3) those designed for attamole or picrogram sensitivity, and (4) those designed for quantitation over a broad dynamic range.

Criterion 2 is often the major factor in assay selection. Antigens with a single epitope can be quantified only by using a competitive configuration and some homogeneous configurations. Multivalent antigens, particularly if they are such a size that steric hindrance is not a problem, can be quantified using any of the configurations.

Routine diagnostic assays, in which time of processing (criterion 4), rather than sensitivity, is more important, can also be a major factor in selection. Some HomEIAs can be completed in a few minutes, although often sacrificing sensitivity. HomEIAs may not be amenable to all antigens. The ELISA-based automated systems designed for clinical laboratories, such as the Abbott TDx and the Ciba-Corning Magic Lite, are generally dedicated for use with a definite panel of human antigens, but not for others, or for those of, for example, veterinary animals. Assays designed for field use, such as screening for herbicide residues in industrialized countries, or detecting hepatitis, typhoid, or parasitic antigens in underdeveloped countries (Talwar et al., 1992), may sacrifice precision and sensitivity for convenience, simplicity, and field stability. A good example of the latter is the so-called dipstick technology, in which antibody-coated NC strips are dipped into potential antigen sources, quickly washed, and then dipped into subsequent solutions containing the detection system needed to complete the assay. In other modifications, the dipstick contains all the reactants at different levels, and the reaction proceeds as the liquid moves up the strip by capillary action.

A factor that may influence the selection of direct (see Fig. 1c) or indirect (see Fig. 1d) competitive ELISAs, is the ability to label the antigen with an enzyme reporter, without seriously altering its antigenicity. When this is impossible, use of an indirect configuration (see Fig. 1d), in which an excess of immobilized capture antigen is used to capture antibody not bound by the solution analyte, can be employed. However, choice of an indirect configuration may be unwise if immobilization of the solid-phase antigen results in an unacceptable loss of antigenically active molecules.

Assay sensitivity can also be a criterion of assay design. The HetEIAs are more sensitive than HomEIA, and the sensitivity of competitive assay is theoretically lower than for two-site assays. In situations requiring very high sensitivity, the use of noncompetitive HetEIAs that employ enzyme-amplification circuits (see Sec. III.B), may be needed.

Sandwich assays may be conducted in two ways, depending on whether the CAb and detection antibody recognize the same or different epitopes. In the former, *asymmetric sandwich*, both analyte and detector can be added simultaneously, as no competition exists for epitopes on the antigen, whereas in *symmetric sandwich* ELISAs, reagent addition must be sequential, although serious steric hindrance can still occur (Butler et al., 1986). Caution is also necessary with simultaneous additions in asymmetric configurations, as inhibition or "hook" effects can result at high antigen input (Porstmann et al., 1983).

There is no single configuration of ELISA that is best for all situations in which antigen must be quantitated. Each is developed and refined for the quantitation of a particular analyte. Although high sensitivity would appear to be an ultimate goal in all good assay designs, its achievement may not be justified by the application (i.e., "why put a 500 dollar saddle on a 5 dollar mule"). The dipstick technology used in India may be a good example of such reasoning (Talwar et al., 1992).

B. Immunochemical Characteristics of ELISAs for Antigen

Figure 7a illustrates a theoretical precision profile (Ekins, 1983) to demonstrate that (1) the measurement error (expressed as the coefficient of variation; CV) is greatest at the extremes of the titration range; and (2) two-site configurations have inherently greater sensitivity and greater dynamic ranges than competitive assays. The capture antibody in a two-site assay is unlimited, whereas the sensitivity of direct competitive assays (see Fig. 1c) is controlled by limiting the number of occupiable sites (i.e., the antibody concentrations). The use of a high antibody concentration will yield an assay that is more precise over the dose range, but in contrast with the same assay performed with a low antibody concentration, it will be less sensitive, i.e. obtaining a signal above background from the enzyme-labeled reporter will require that a larger amount of unlabeled competitor be added (see Fig. 7b; low vs high). Thus, antibody concentration determines both the working (dynamic) range and the LOD in competitive ELISA (see Fig. 7b).

Although the number of available sites on immobilized CAb in a two-site (sandwich) configuration is limited only by the capacity of the solid phase, detection of low concentrations of

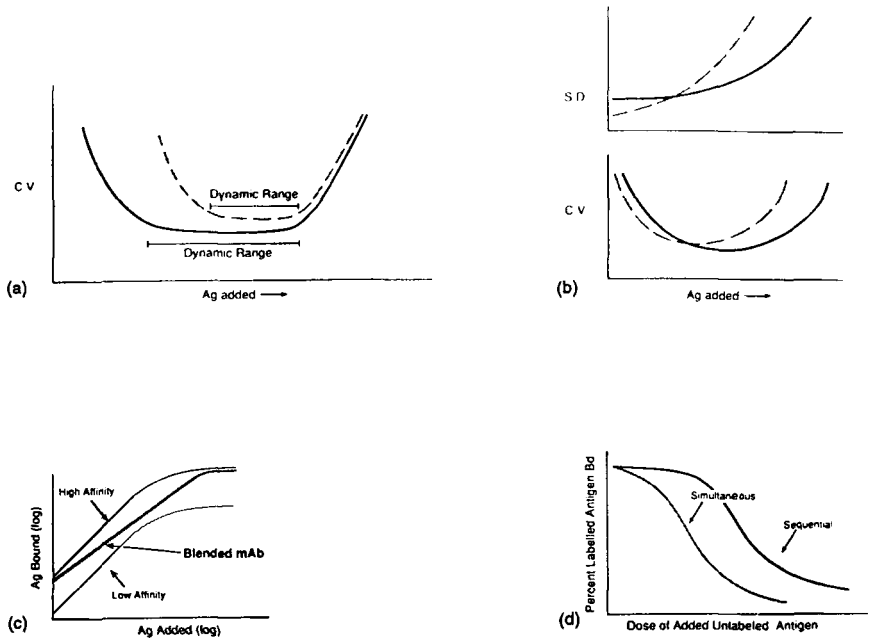


Figure 7 Precision profiles and related graphic methods for expressing the sensitivity and working ranges of ELISAs. (a) Precision profile of sandwich (solid line) versus competitive (broken line) ELISAs; (b) Precision profiles in a competitive ELISA using low (broken line) and high (solid line) antibody concentrations. (c) Effect of using blended monoclonals on the dynamic range of ELISAs. (d) The effect of simultaneous versus sequential addition of labeled and unlabeled antigen in a direct competitive ELISA. [(b) from Ekins, 1983.]

analyte can be seriously compromised by NSB. In Fig. 1b, the number of reactants that can short-circuit to the solid-phase CAb in the absence of any analyte (see Fig. 2) is proportional to the complexity of the detection system (amplified or nonamplified; see Fig. 1b). This problem worsens as more protein (i.e., CAb) is immobilized and the potential for NSB increases. It is believed that the Fc portions of CAb and detection antibodies play a substantial role in NSB; hence, the use of Fab fragments appears to reduce NSB.

The affinity of an antibody for the ligand (K_{eq}) also affects assay performance. A comparison of sandwich assays performed with antibodies of different K_{eq}, shows that high-affinity antibody quickly becomes saturated, but is able to give a considerable signal above background for low doses of added analyte (see Fig. 7c). Because the situation is reversed for low-affinity CAbs, polyclonal CAbs or deliberately blended monoclonals, often provide the optimum in sensitivity and dynamic range (see Fig. 7c).

Competitive assays are performed in various permutations. Many of the described variants, such as the use of an adsorbed antiglobulin to immobilize the mouse CAb (e.g., goat antimouse), rather than direct immobilization of the CAb (see Fig. 1e vs Fig. 1c), represent only a variation in the method of CAb immobilization, but not a fundamental change in immunochemical principle. However, the indirect configuration shown in Fig. 1d is a variant that also affects immunochemical principles. Indirect competitive ELISAs do not require the labeling of the analyte; thus, they offer a universal approach to the design of assays for quantitation of univalent antigens. Namely, antibodies are stable proteins, and the same procedure can be used to label all of them, whereas antigens must be individually treated, and each can offer a new technical challenge during conjugation. Direct competitive assays also differ fundamentally, depending on whether the labeled and unlabeled analyte are simultaneously added as opposed to sequentially added. When the unlabeled ligand, over a range of concentrations, is first allowed to bind antibody at equilibrium, and then the labeled ligand is added, establishment of the new equilibrium will depend on the k_2 or off-rate, and will be proportional to the antibody K_{eq}. If the amount of bound, labeled ligand is determined before the new equilibrium has been established, the LOD of the assay will be lowered (see Fig. 7d).

Equations (1) and (2) demonstrate the importance of eliminating NSB to improve assay sensitivity and Sec. II pointed out the contribution which the solid phase brings to this problem. If surfaces cause problems, especially NSB, why not eliminate them? Such is the rationale behind the HomEIA. Among the best-known is the EMIT system (Ullman et al., 1979). Digoxin can be quantified by coupling a standard to a reduced mAb specific for glucose-6-phosphate dehydrogenase. There are also substrate-based HomEIA. Glucose oxidase requires interactions with flavin-adenine dinucleotide (FAD) for full activity. Complexes of FAD and the analyte inhibit the enzyme so that increasing the free analyte in a solution-phase competitive assay will release more FAD–analyte to inhibit the enzyme.

In general, dehydrogenases and hydrolases are most widely used for HomEIA, and drugs (e.g., digoxin) have been among the most common analytes. A variety of ingenious systems have been devised, many similar to the examples given, but also including steric hindrance models (Castro and Monji, 1985) enzyme-channeling immunoassay (Litman et al., 1980), and those that signal through an electron-transfer reagent, such as ferroscein.

Although HomEIA has eliminated the NSB associated with the solid-phase as well as introducing single-step versus multistep assays, several immunochemical (as well as practical) problems still exist. First, the time saved in HomEIA, by eliminating the need to separate bound and free reactants through sequential washings and reagent addition steps, means that unreactive components in the specimen remain present to cause the "serum interference" that characterizes HomEIA. As interference is a function of concentration, HomEIA works best for analytes in high

concentrations that allow high dilutions of serum to be tested and are least effective for low analyte concentrations requiring nearly undiluted specimens. Second, HomEIA is a competitive system, thus possessing the same limitations in dynamic range and sensitivity that characterize competitive configurations in general (see Fig. 7a). Finally, not all antigens are amenable to a system that involves their conjugation to enzymes, substrates, or cofactors, the actions of which they must modify to produce a functional assay.

C. Enumeration of Antigen-Secreting Cells (ELISPOT)

Enumerating the proportion of in vitro-cultured lymphocytes capable of secreting antibody has been a prime tool of immunobiologists; this was done for more than 20 years using the hemolytic or "Jerne" plaque assay (Jerne et al., 1974). Although this method historically contributed a great deal toward understanding the process of B-cell activation and the clonality of the response, the assay relied on a complex chain of events that involved either a primary (direct Jerne plaque) or secondary (indirect Jerne plaque) activation of complement sufficient to cause lysis and produce clear plaques surrounding antibody-secreting cells in soft agar. As reviewed by Czerkinsky et al. (1988a), interpreting such data can be difficult and has been periodically questioned, especially concerning the affinity of the secreted antibody (DeLisa, 1976).

Czerkinsky and co-workers (Czerkinsky et al., 1983a) and Sedgwich and Holt (1983) simultaneously adopted ELISA technology to the more direct measurement of antibody or antigen secretion by lymphoid cells in vitro. The principle is the same as the one on which other HetEIAs are based; either antigen or antibody adsorbed to polystyrene serves to capture the analyte of interest. The initial invention involved adsorption of the capture reagent to the surfaces of plastic tissue culture laboratory ware. Lymphoid cells, akin to those used in Jerne plaque assays, are then added and allowed to assume sessile secretory positions for about 4 h. Antibodies secreted by such cells are captured by adsorbed antigen. Secreted antigens (e.g., immunoglobulin) can be captured by solid-phase CAbs. After removal of the cells, the site of secretion is visualized by the addition of an enzyme-labeled antibody in the manner of a typical two-site or sandwich ELISA (see Fig. 1b) or SpAbI (see Fig. 1a). However, the substrate used for either HRP (OPD) or AP (5-BCIP), which is dissolved in agarose (to reduce diffusion), is one that produces local deposition of an insoluble product. Focal accumulation of such products as the agarose solidifies, produces spots or ELISPOTS, at those sites where a secreting cell had been present. The method has broad application for estimating the number of cells in any suspendable population that can secrete an antigenic product that can be captured by an immobilized CAb. The technology can be expanded to two-spot assays, using separate capture and detection systems, that produce substrate products with different colors (Czerkinsky et al., 1988b). Furthermore, the ELISPOT test can be performed on other solid phases, such as on NC (Möller and Borrebaeck, 1985). The use of NC with its high-adsorptive capacity, resolved a curiosity of ELISPOTs performed on plastic. By using a plastic solid phase, very large amounts of antigen (100 μg/ml), especially if of low molecular mass, are needed (Holt et al., 1984). Furthermore, the use of NC eleivates the need to incorporate agarose into the substrate. Another modification involves Immobilon-P, rather than NC, to directly adsorb the secreted product at the site of secretion (Kendall and Hymer, 1987).

Specifically for immunologists, the method readily permits the identification of either secreted immunoglobulins or antibodies down to the level of their subclass, allotype, and idiotype. This would be a very difficult, if not impossible, task using the hemolytic plaque assay. The ELISPOT also allows parallel sets of culture wells (or small petri dishes) to be developed (1) one set uses conventional product deposition to enumerate the *number of secreting cells*

whereas (2) the other set employs a substrate that yields a soluble product that can then be measured spectrophotometrically to determine the *total amount* of secreted antigen or antibody.

The use of ELISPOT technology has also been extended to accessing cell proliferation. Rather than using tritiated thymidine, cells are allowed to incorporate the pyrimidine analogue 5-bromo-2′-deoxyuridine (BRDU), which is subsequently detected using an mAb specific for BRDU (Porstmann et al., 1985; Magaud et al., 1988).

D. Enumeration of Antigen-Bearing Cells (Cell-ELISA)

The localization of antigens in tissue secretion using enzyme-labeled antibodies, dates to the late 1960s (Nakane and Pierce, 1966; Sternberger et al., 1970). With exception of a single early report (Avrameas and Guilbert, 1971), this method was routinely employed only a decade ago for detection of surface antigens on cells in suspension (Douillard et al., 1980; Lansdorp et al., 1980; Suter et al., 1980). Cell-ELISAs or CELISAs, are performed in 96-well microtiter plates, often as simple inexpensive plus–minus screening assays, and often as a rapid preliminary to more expensive fluorescein-activated cell sorter (FACS) analyses. Cell-ELISAs appear to be as sensitive as FACS (Aida et al., 1987).

A critical step in the performance of cell-ELISAs is the immobilization of the cells, most often those of the lymphoid type, to the microtiter well surface. The common methods used are similar to those described in Sec. II for bacterial, viral, and protein immobilization. These include the use of an antilymphocyte CAb or glutaraldehyde (Stocker and Heusser, 1979), or adsorption of poly-L-lysine, followed by glutaraldehyde cross-linking (Lansdorp et al., 1980; Suter et al., 1980). Alterations in cell surface antigens or increases in NSB through the use of glutaraldehyde, prompted the viable cell adaptation of Posner et al. (1982). Although the same detection systems as those described in Sec. III are used, HRP and AP can be problematic owing to the presence of endogenous peroxidases and phosphatases. Therefore, the use of βgal (Cobbold and Waldmann, 1981; Borzini et al., 1981) as a signal generator, together with the viable cell procedure just described, is perhaps the current method of choice (Sedgewick and Czerkinsky, 1992).

Cell-ELISAs have been applied to a variety of cells and cell antigens (see review by Sedgewick and Czerkinsky, 1992), including the major cell antigens of interest to immunologists (e.g., MHC-I, MHC-II, β_2-microglobulin, IgM, Thy-1, Fc-receptors, tumor cell markers, IL-2 receptors, and numerous CD markers). Cell-ELISA has also been employed to study the internalization of surface immune complexes (e.g., "caps"; Yokota et al., 1989) and is adaptable to the use of fluorescent signal generators when fluorometric plate readers are available (Avner et al., 1988).

E. Immunoblotting or Western-Blotting ELISA

The concept of transferring biomolecules separated in one medium (e.g., agar or acrylamide) to adsorptive membranes, was pioneered by E. M. Southern (1975) for the detection of DNA hybrids. Scientists involved in immunochemical characterization of proteins soon realized the obvious benefits of transferring proteins from high-resolution polyacrylamide gels to an immobilizing membrane for subsequent specific immunochemical detection (Renart et al., 1979; Towbin et al., 1979). This form of SPI follows many of the same principles and uses many of the reagent configurations and detection methods as those used in ELISAs. In addition, immunoblotting is particularly suited to the use of amplified detection systems such as chemiluminescent (ECL, Amersham; Western Light, Tropix; Lumi-Phos, Boehringer-Mannheim), CARD (BLAST, DuPont NEN), and immune complex amplification (Pluzek and Ramlau, 1988). Immunoblotting is described in detail in Chap. 35 of this book.

V. SPECIFIC ANTIBODY IMMUNOASSAY

A. Quantitation of Antibody Versus Antigen

The measure of specific antibody to an antigen is, simplistically, the reversal of an assay in which antigen is the dependent variable. However, this simple reversal is complicated because, although antigens to be quantified are antigenically homogeneous, serum antibodies are neither homogeneous in terms of the antigenic epitopes they recognize nor in their affinity (K_{eq}) for these epitopes. Hence, quantitation of specific antibodies in absolute units (μg/ml) is much more complicated than quantitation of antigen in the same units. Thus, over the years, antibody quantitation has been expressed as *titer* or *units of activity*, rather than micrograms per milliliter. These measures of activity reflect both the amount present and its affinity (see Sec. V.D). Our laboratory and others (Butler and Hamilton, 1991) have adopted the abbreviation SpAbI to emphasize that such assays are designed to measure antibody; this seems less ambiguous than the term *antigen-specific ELISA* that has often been used. It is assumed that anyone wishing to measure antibody plans to measure antibody that binds a *specific antigen*. Thus "antigen-specific" is both redundant and confusing.

The immunochemical differences between antibody and antigen quantitation can be summarized as follows:

1. Serum antibodies (monoclonals excepted) are *heterogeneous* in epitope recognition and affinity. This means that affinity or epitope bias, can significantly influence titer. In contrast, ELISAs configured to measure antigen employ a single antibody preparation to quantify a homogeneous antigen.
2. Antibodies are also heterogeneous in terms of their class or subclass [i.e., they possess different constant (C−) regions]. Therefore, antibody detection in an SpAbI (see Fig. 1a) depends on the specificity of the antiglobulin (mouse mAb in Fig. 1a) to recognize a single subclass of antibody or to recognize all classes equally.
3. The detection of minor classes (e.g., IgE) can be seriously compromised by IgG antibodies recognizing the same epitope and present in 100- to 1000-fold greater amounts.

The combined effect of the afore described factors emphasizes that unless very special immunochemical configuration are employed, ELISA-based SpAbIs are best considered as a measure of antibody *activity*, rather than the absolute amount present.

B. Antigen Selection and Method of Immobilization

Section II of this chapter described the complication to SPI, which results from adsorption-induced conformational changes in proteins. The capture of only a small proportion of total antibody by globular protein antigens adsorbed on plastic (see Fig. 5) may be the consequence of such changes. Awareness of this phenomenon has given rise to various alternative means of antigen immobilization (see Sec. II.B–E). Discussions in these latter sections provide examples of methods that have been successfully used to preserve antigenic integrity. Because of the many differences among potential antigen and our relatively poor understanding of the factors that alter antigenicity during immobilization, it is impossible to suggest a universal method of immobilization. Instead, one must revert to philosophy. Hence, the best method of immobilization must be empirically determined and should be the one in which the immobilized antigen retains the highest proportion of the antigenicity that it expresses in solution. How is this tested? First, employ an immunochemical principle common to all immunoassay; inhibit the solid-phase interaction with the soluble antigen. If the logistics can be solved, attempt inhibition of the solution-phase interaction with the solid-phase antigen. As a consequence of antigen

alteration, SpAbI ELISA titers may not agree with serological titers by other methods. Hendriksen et al. (1988) showed that, although ELISA-based SpAbI for antitetanus correlated poorly with in vivo toxin neutralization tests, an inhibition ELISA assay in which the ability of sera to inhibit the binding of tetanus toxoid to antitoxin-coated plates, correlated well with in vivo neutralization.

C. Competition, Parallelism, and Heterogeneity

Figure 8a illustrates a typical ELISA SpAbI titration plot. When the results are expressed as titer, this is often done either in terms of *endpoint* (i.e., the highest dilution giving a signal above the control; the LOD), or as *midpoint* titer. The latter is a particular signal or report that falls in the midpoint of the linear titratable region. The SpAbI titrations, when performed as HetEIAs, often show a so-called hook or prozone effect at low serum dilution. This can be caused by various factors, including (1) the preferential dissociation of large immune complexes; (2) the preferential binding of smaller, less enzymatically active antibody–enzyme conjugates to densely packed antibodies, which sterically hinders the binding of larger conjugates; (3) inhibition of enzymatic activity through steric hindrance; and (4) competition among antibodies of different isotypes for the same antigenic determinants. The latter frequently occurs in microtiter ELISAs when antibodies of a minor isotype are being measured in serum containing antibodies of a major isotype that recognize the same determinants. Figure 8b illustrates this phenomenon in the detection of rabbit IgA antibodies. Titration plot B is that for IgA antiovalbumin (OA) in rabbit serum before removal of IgG with *S. aureus* (plot B′; see Fig. 8b). Removal of competing IgG both increases the titer of IgA anti-OA and removes the hook or prozone effect.

The negative consequences of competition can be circumvented by treating the specimen beforehand to remove or reduce the competitor (see Fig. 8b) or through assay designs in which the immunoglobulin of the isotype to be measured is first specifically captured, and then the antibody activity of the captured immunoglobulin is measured (see Fig. 1e). Competition is especially problematic for detection of antibodies belonging to minor serum immunoglobulin classes (e.g., IgE and IgA).

Figure 8b also illustrates another important feature of HetEIA that is often seen with SpAbI. Namely, the lack of parallelism among the titration plots of specimens being compared. Plotted in a log–log fashion, two-site HetEIA for antigen (see Fig. 1b) or antibody measurement (see Figs. 1a and 8b), should produce a log-linear titratable region, with a log–log slope of approximately 1.0. Deviations from this represent competition, NSB, or both (Butler and Hamilton, 1991). Plot A of Fig. 8b is that of the reference standard (RS) and has a log–log slope of 0.964; only plot B′ in which competitive IgG has been removed, yields a similar slope. The slope of plot B is flatter than the RS owing to the competitive effect just described, whereas the flatter slope of plot C results from the high NSB, which is most pronounced at high serum dilution.

The measurement of serum antibodies by SpAbI in HetEIA means measurement of polyclonal antibodies (i.e., those derived from many different clones that differ in both their epitope specificity and their affinity). What effect does such heterogeneity have on a SpAbI titration plot? Figure 7c, although designed to illustrate the effect of antibody affinity in antigen quantitation, is also applicable to SpAbI merely by changing the units of the x-axis from "Ag added" to "Ab added." Monoclonal antibodies give linear log–log titrations, with affinity differences affecting only the y-intercept. Heterogeneous populations (e.g. "blended in mAbs"; Fig. 7c) give nonlinear titration plots, with progressively decreasing slopes as more antibody is added. As most SpAbIs are performed on polyclonal sera, such heterogeneous titration plots are the rule in most research. However, two points are noteworthy: (1) one or several high-affinity populations in a polyclonal

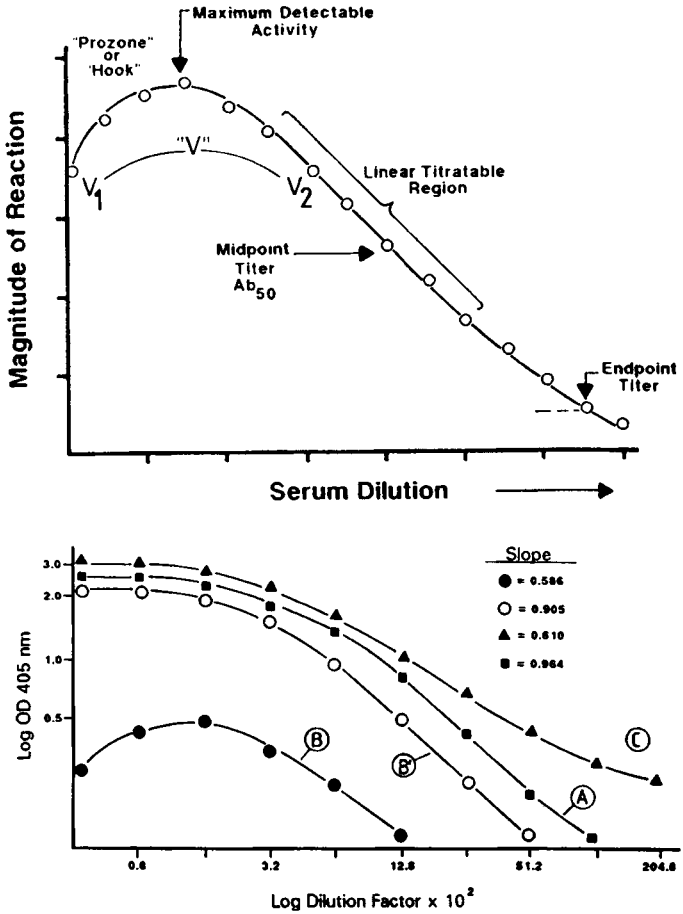


Figure 8 The anatomy of a titration plot: (a) A typical titration plot, showing such features as prozone, the titratable region, maximal activity, and endpoint titer. V_1 and V_2 illustrate two points that generate an equal signal, but cannot be distinguished by single-point determination. (b) Factors that influence parallelism in titration plots: Plot A, the proper titration of an SpAbI when expressed in log–log fashion has a negative slope of approximately 1.0; plot B, the titration of IgA anti-OA in rabbit serum in which extreme prozone inhibition is observed, as well as a titration slope of significantly <1.0; plot B′, the titration of IgA anti-OA in rabbit serum after partial depletion of IgG anti-OA (Butler et al., 1990); plot C, a titration plot with decreased slope caused by nonspecific binding at high sample dilution.

sera can dominate the titration, making it appear monoclonal; and (2) many investigators plot their titration semilogarithmically making interpretation in the foregoing manner especially difficult. For further discussion of these effects, see Peterman (1991a).

D. Units of Antibody Measurement and Data Expression

The concepts of titer and the anatomy of a SpAbI titration plot were discussed in the foregoing. Although titer represents a generally well-accepted method of expressing data from SpAbIs, it can result in a number of ambiguities. First, titers (whether midpoint or endpoint) represent single-point determinations and are valid only when the titration plots being compared are parallel (see Fig. 8b). Hence, titers can be in error owing to the many factors that cause hooks, prozones, and other types of nonparallelism (see foregoing Sec. V.C). Second, when titer is used, it is best to relate it to some RS tested on the same microtiter plate and on the same day, When such RSs are not shared among laboratories, titer values (often expressed as the reciprocal of the serum dilution at the LOD) are rather a "private" form of data expression from a laboratory. To appreciate the contrast with antigen measurement, consider the task of summarizing the data of various laboratories for antigen expressed in micrograms per milliliter versus serological data expressed as titer.

The ambiguities associated with titer are perhaps best resolved by a combination of multipoint analyses and RS expressed in units of activity per milliliter (Butler and Hamilton, 1991). One method of multipoint analysis is ELISANALYSIS (Butler et al., 1985; Butler, 1988; Peterman and Butler, 1989; Peterman, 1991b), which is based on the principle that titration plots expressed in log–log fashion give a titratable region (see Fig. 8a) with a log–log near 1.0 (see Fig. 8b). Therefore, ELISANALYSIS simply compares the various titrations for SpAbI over the log–log titratable region to (1) check for parallelism and (2) generate a value in activity units for each dilution of the unknown tested, compared with the RS run on the same microtiter plate. Figure 9 gives a typical computer printout for ELISANALYSIS during measurement of human IgA antibodies to *Streptococcus mutans*. Below the standard curve, the numerical values for the RS are given including the activity units (EU/ml) at each point. In this example, the RS was designated to contain 100 EU/ml. The printout then treats four dilutions of each unknown in the same manner. Inspection of the data for the unknown reveals that the serum of patient 1 does not titrate parallel to that of the RS; rather each EU per milliliter value increases with dilution. The remaining three patients all give parallel titrations for at least three consecutive dilutions. All of these manipulations are performed using a PC interfaced to a suitable microtiter plate reader. A number of modern plate readers contain built-in curve-fitting routines, including some modeled after ELISANALYSIS.

The use of ELISANALYSIS and other multipoint analytical systems require that unknowns be tested over a range of dilutions; single-point testing is considered unreliable. Even though this section is devoted to SpAbI, multipoint analysis is applicable to all forms of HetEIA, although other forms of curve-fitting (e.g., four-parameter, polynomial, or other) may be more appropriate for some competitive configurations (see Peterman, 1991a).

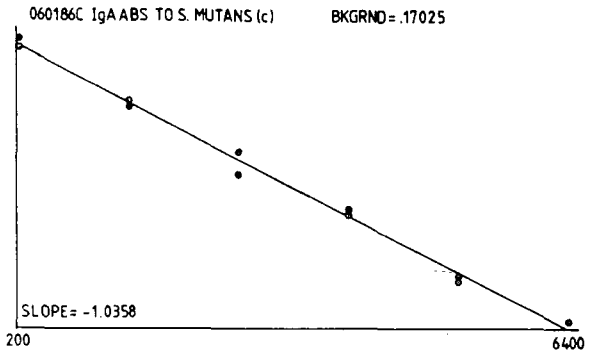
Although multipoint testing generates more reliable and comparative data, the issue of ELISA units per milliliter (or other activity measurements) requires discussion. As mentioned at the beginning of Sec. V, a major difference between antigen and antibody quantitation lies with the units of measurement. Antigens are homogeneous in their binding to the antibody used to measure them (i.e., the proportion of the added antigen that binds for a given dose is constant and is determined by the K_{eq} of the interaction). Therefore, values for unknown specimens can be directly interpolated from a standard curve made with an RS of the antigen in micrograms per

060186C IgA ABS TO S. MUTANS(c)

LN/LN plot
Equation of the line: Y = -1.035758 * X + 5.456356
Correlation Coef.= .9969003 for N= 11

Background set at .17025

For Dilution Range 200 to 6400



060186C IgA ABS TO S. MUTANS(c)

#	O.D.	D.F.	Equiv. D.F.	E.U./ml	On Curve?
1	1.132	200	201.472	99.26959	
2	1.228	200	183.789	108.8205	
3	.651	400	393.514	101.6481	
4	.617	400	422.392	94.69076	
5	.421	800	737.701	108.445	
6	.361	800	960.631	83.27863	
7	.285	1600	1569.094	101.9697	
8	.294	1600	1458.775	109.6811	
9	.219	3200	3585.855	89.23952	
10	.223	3200	3322.974	96.29928	
11	.2	6400	5776.65	110.7909	
12	.178	6400	21168.715	30.2333	Below

060186C IgA ABS TO S. MUTANS(c)

#	O.D.	D.F.	Equiv. D.F.	E.U./ml	On Curve?
25	1.187	100	190.939	52.37266	
26	.727	200	341.523	58.5612	
27	.564	400	477.162	83.82891	
28	.436	800	697.46	114.702	

060186C IgA ABS TO S. MUTANS(c)

#	O.D.	D.F.	Equiv. D.F.	E.U./ml	On Curve?
45	.582	1000	457.007	218.8148	
46	.427	2000	721.05	277.3733	
47	.292	4000	1481.905	269.9228	
48	.189	8000	9020.707	88.68484	Below

218.8148
277.3733
269.9228
Mean= 2.55E+02 for 3 points
Variance(N-1)= 1.02E+03
Std Error= 1.64E+01
Coeficient of Variation= 10.2%

060186C IgA ABS TO S. MUTANS(c)

#	O.D.	D.F.	Equiv. D.F.	E.U./ml	On Curve?
65	1.368	400	163.005	245.392	Above
66	.743	800	332.308	240.7409	
67	.44	1600	687.472	232.7368	
68	.332	3200	1126.43	284.0834	

240.7409
232.7368
284.0834
Mean= 2.53E+02 for 3 points
Variance(N-1)= 7.63E+02
Std Error= 1.59E+01
Coeficient of Variation= 6.9%

060186C IgA ABS TO S. MUTANS(c)

#	O.D.	D.F.	Equiv. D.F.	E.U./ml	On Curve?
77	.599	20	439.501	4.550617	
78	.434	40	702.565	5.693421	
79	.293	80	1470.247	5.441262	
80	.229	160	2994.724	5.34273	

4.550617
5.693421
5.441262
5.34273
Mean= 5.26E+00 for 4 points
Variance(N-1)= 2.44E-01
Std Error= 2.47E-01
Coeficient of Variation= 8.1%

milliliter. By contrast, the antibodies in a serum specimens are heterogeneous in their K_{eq}, vary from one specimen to the next, and are unlikely to have the same K_{eq} as the serum used as a RS. Hence, the proportion of antibodies in an unknown specimen that binds when a particular dose is added, will differ from serum to serum and from that of the RS. Therefore, although one may be able to express the results in terms of micrograms of *bound antibody* relative to a RS (provided the amount bound in the RS is known), it would be incorrect to express it as micrograms per milliliter of antibodies because the fraction of antibody free at equilibrium is unaccounted for.

This problem, therefore, has been resolved merely by assigning units of activity to the RS used to measure the antibody. Obviously, activity reflects both the amount and the K_{eq} of the antibody and may be most indicative of the functional role of the antibody in vivo, as opposed to absolute gravimetric units. This issue is discussed in more detail by Butler and Hamilton (1991), including the various means that have been used to circumvent this difficulty and to express antibody in absolute units.

Finally, one situation exists in which expression of absolute units of antibody is valid and appropriate. This involves in vitro culture experiments in which mAbs are being quantified using known amounts of the same mAb as an RS.

E. Complications in the Use of Specific Antibody Immunoassays

Several of the complicating factors in ELISA-based SpAbI have already been discussed, including (1) the effect of competition on the accurate measurement of antibodies of minor isotypes, (2) the difficulty in comparing data among laboratories when expressed as titer, (3) the dangers of single-point (e.g., endpoint) determinations, (4) the danger of expressing antibody levels in gravimetric units, except in special situations, and (5) complications that can result from antigen alteration during immobilization. In addition, several additional complications warrant discussion.

Assay bias caused by epitope presentation can result from epitope loss during immobilization (e.g., epitopes recognized by IgE are lost, but not those recognized by IgG). In addition, immunodominant epitopes, which may comprise only a small proportion of epitopes on a multivalent antigen, may be recognized by many antibodies with high affinity. Hence, the titer or activity of a serum toward a complex antigen may be heavily skewed by antibodies that recognize only a few epitopes.

Complications can also involve the detection system, especially the isotype- or subisotype-

Figure 9 Multipoint analyses of ELISA data illustrated by ELISANALYSIS. Computer printout of the measurement of IgA antibodies to *Streptococcus mutans*. The regression analysis of the log–log titration of the reference standard is given in the upper left-hand region (060186C references the date and microtiter plate). The numerical values for six different dilutions (in duplicate) of the reference standard are given below the log–log plot; the columns left to right are plate well no., OD405, dilution factor (D.F.), the computer-determined actual dilutions (Equiv. D.F.) and further to the right, the calculated E.U./ml for each dilution of the reference standard. ELISANALYSIS corrects for dilution so that the mean of these values should be 100 EU/ml for the reference standard. The same analysis is applied to test samples, and data are presented in the same format on the right side of the figure for four different samples. Values which fall "above" or "below" the reference standard plot are accordingly "flagged" in the column entitled "on curve?" When the values in EU/ml are independent of sample dilution (last three test samples shown), the investigator can be sure that the test sample is titrating with the same slope as the reference standard (see Fig. 8b). When three or more consecutive values fall within the reference standard curve and give values indicating that they titrate with the same slope, ELISANALYSIS computes the mean and appropriate statistics for such samples (exemplified in a box for the last sample tested).

specific antiglobulin (see Fig. 1a). In cattle, the A1-allotype is an immunodominant epitope recognized by nearly all polyclonal and monoclonal antibodies prepared against IgG2a, whereas the A2-allotopes are poorly recognized (Butler and Heyermann, 1986; Butler et al., 1993). Hence, when antibodies to IgG2a in cattle are measured by SpAbI, caution is needed to be sure that titers do not differ merely because of the allotype of the animal tested.

Complications involving antiglobulin specificity can be especially problematic when using mAb specific for various human IgG subclasses (Butler and Hamilton, 1991). Difficulties in this area will require that recombinant DNA technology be increasingly employed to prepare matched panels of engineered subclass proteins and their specific mAbs or mAb "blends."

Serum is a highly regulated body fluid, whereas exocrine secretions are not. The latter are subjected to a variety of dilution effects so that the expression of antibody activity in EU per milliliter in the field of secretory or mucosal immunity may be very misleading if no control for physiological dilution is employed. A variety of methods have been used or proposed, of which nearly all involve comparing the activity in units per milliliter with the concentration of a specific protein, such as albumin, or with the total protein content of the fluid.

The use of complex antigen mixtures on the same solid phase in SpAbI can result in misleading data for reasons such as epitope bias, competition, and steric hindrance. Therefore, allergy test systems such as MATRIX (Abbott Laboratories), in which extracts of potential allergens are distributed on separate portions of a solid phase, seem more feasible. Ultimately, such approaches could lead to the *microspot* technology described by Ekins and co-workers (Ekins, 1991).

VI. NUCLEOTIDE-ELISA: THE PHOSPHORUS 32 AND SULFUR 35 ALTERNATIVE

The rapid progress in medical science in the last two decades has been largely due to the development, or refinement, of a handful of biotechnologies (e.g., recombinant DNA technology, monoclonal antibody technology, HPLC, and ELISA or SPI). More important is that each of these techniques complement the other and are often used together. In the 1970s, ELISA technology began to supplant RIA as enzymes became the preferred signal generators for SPI. If this was possible, why not use enzymes as reporters for hybridization in molecular biology? The ^{32}P-nucleotides have a $t_{1/2}$ of 14 days and, in the last year, the University of Iowa alone purchased >1.6 million dollars of both ^{32}P- and ^{35}S-labeled reporter nucleotide phosphates. National statistics on this are not readily available, nor are figures on the annual costs resulting from the handling and the waste-disposal associated with the use of these reporters, but, no doubt, it would be substantial.

The application of ELISA technology to molecular biology is neither novel nor new. Kourilsky et al. proposed such an application in 1978, contemporary to the time when HetEIA was replacing RIA. A variety of methods were then described in the 1980s. Up to this point, each of these nucleotide-ELISAs (N-EIA) required that the nucleotide probe be chemically modified, with the potential that such modifications could disturb the probe's hybridization properties. As most nucleic acid hybridizations are performed as solid-phase assays on nylon or NC membranes, the problem of NSB encountered in immunoblotting (see Sec. IV.E) cannot be overlooked, and methods used to reduce NSB in protein–protein interaction could affect the stability of hybridized nucleic acid duplexes. Thus NSB can be expected to be a problem in N-EIAs.

The most common procedures for the labeling of nucleotides involves labeling with haptens, such as biotin (Kourilsky et al., 1986), dinitrophenol (Vincent et al., 1982), 5-bromo-2′-deoxyuridine (Traincard et al., 1983), sulfone groups (Lebacq et al., 1988), digoxigenin (Kessler et al., 1989), and acetylaminofluorene (Tchen et al., 1984). Probes may be directly conjugated, or

biotin and other haptens may be incorporated during the polymerase chain reaction (PCR; Lo et al., 1988). Hapten-labeled probes are either detected with specific mAbs that recognize them, or by using streptavidin–enzyme. As discussed in Sec. I and III, the more complex the detection system in terms of protein constituents, the greater the potential for NSB. Nonspecific-binding can be further intensified if the blotted material contains biotin-binding or carrier proteins (Zwadyk et al., 1986), or if endogenous enzymes are transferred (see Sec. III). When AP is used, it is recommended that filters be baked at 80°C to inactivate endogenous AP.

The LOD of simple colorimetric detection systems may be too insensitive for most N-EIAs. However, both the cyclic amplification system of Self (1985) and the CARD system (see Sec. III) appear to be effective (Gatley, 1985). Most N-EIAs now rely on chemiluminescence (CL) for detection. As described in Sec. IV.E, the latter also offers the advantage of reprobing the same blot. The CL detection systems for N-EIA are marketed under various trade names [e.g., Lightsmith (Promega), ECL (Amersham), and Genius (Boehringer-Mannheim)]. The enzyme and chemiluminescent substrates used in these respective systems are AP–AMPPD, HRP–Luminol, and AP–Lumigen PPD (see Table 3). The Lightsmith system differs from the others in that oligoprobes are amino-modified and then directly coupled to AP (Jablonski et al., 1986; Pollard-Knight et al., 1990). The manufacturer claims that this eliminates the NSB problems that are often encountered with N-EIA; furthermore, it is claimed that 20- to 40-fold higher signals are obtained with these probes, compared with ^{32}P-oligos. The probes can also be used in dideoxy sequencing.

Alternatives to ^{32}P- and ^{35}S-oligonucleotides originally received slower acceptance in the United States than in Europe. In Switzerland, where the cost of ^{32}P is fivefold higher than in the United States, change to N-EIA was more rapid (Suter, M., personal communication). In any event, the pace of technological development has been such that by the time this book is printed, newer methods for N-EIA will have undoubtedly appeared and N-EIA may be the principal method used in molecular biology in the United States of America.

ACKNOWLEDGMENT

The author thanks Dr. Imre Kacskovics for preparation of many of the diagrams and cartoons used. The author is also indebted to Ms. Marcia Reeve for preparation of the typescript.

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