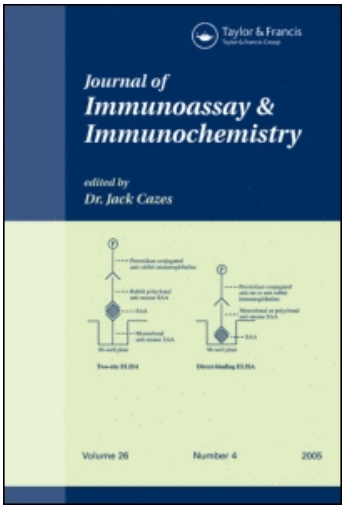

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The Recognition of Proteins and Peptides by Antibodies

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I. INTRODUCTION

Immunological recognition is mediated by T and B lymphocytes and by immunoglobulin molecules secreted by plasmocytes. The T cells recognize protein antigens by means of their T-cell receptors (TCRs) after the antigen has been processed into peptide fragments, a topic discussed in Chapter 13. The B cells recognize antigens by means of B-cell immunoglobulin receptors, which are actually antibody molecules anchored in the B-cell membrane. Unlike T-cell receptors, B-cell receptors are able to recognize the native tertiary structure of a protein antigen. The antibody molecules that are subsequently released by the B cell, after its differentiation into a plasmocyte, possess the same specificity as the receptors of the triggered B cell; therefore, they are also able to recognize the native conformation of the protein antigen.

The first selective theory of antibody diversity was developed at the beginning of the century by Paul Ehrlich, who proposed his "side chain" theory to explain the appearance of specific antibodies. According to this theory, lymphocytes possess on their surface a variety of side chain groups or receptors that are able to combine in a specific manner with different antigens. The interaction of the antigen with one of the side chains was believed to result in the release of that side chain group from the cell surface and to trigger the subsequent synthesis and release of large numbers of the same side chains. According to Ehrlich, the antigen selects, on the surface of the lymphocyte, a receptor with a complementary shape, and it induces the cells to excrete large numbers of this receptor in the form of specific antibodies. This side chain theory is remarkably modern, since it is similar to the currently accepted clonal selection mechanism, which links the specificity of antibodies appearing in the serum with the presence of preexisting clones of cells possessing the same antibodies immobilized at the cell surface.

The molecular mechanisms of antigen recognition by an antibody anchored in the B-cell membrane or by a free antibody molecule are the same. However, it is much easier to study the binding of antigens to free antibodies than to antibodies attached to B cells, and most of our knowledge concerning antigen–antibody binding has been derived from studies with free antibody molecules.

The term *antigen* refers to any entity that is able to generate an immune response in higher vertebrates and to be recognized by the products of the immune response. The ability of antigens to react specifically with complementary antibodies is known as *antigenic reactivity*, and their

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ability to induce an immune response in a competent host is known as *immunogenicity*. This property of immunogenicity depends on the potentialities of the host being immunized, such as its immunoglobulin gene repertoire and various cellular regulatory mechanisms, and it has no meaning outside the host context (Berzofsky, 1985).

The antigenic reactivity of a protein resides in restricted parts of the molecule known as antigenic determinants or *epitopes*. Each epitope corresponds to a cluster of amino acid residues that binds specifically to the binding site or *paratope* of an immunoglobulin molecule. The epitope nature of a cluster of residues in a protein can be recognized only through the binding of an immunoglobulin. Likewise the antibody nature of an immunoglobulin becomes established only after its complementary antigen has been identified. An epitope, therefore, is not an intrinsic feature of a protein existing independently of its paratope partner, but is an entity that can be defined only operationally by the binding of a complementary paratope (Van Regenmortel, 1989). Both epitopes and paratopes are thus relational entities defined by their mutual complementarity. Like husbands and wives, they need each other for achieving the status of partner, and it makes no sense to analyze their properties outside of this relational nexus. Furthermore, since the occurrence of a binding reaction is required for identifying the interacting partners, it is the functional activity during the binding process, and not the static structure of a cluster of atoms, that makes it possible to define each of the two partners. In other words, the concept of epitope is derived from the process of binding to a complementary partner, and not from a structure that could be defined before the interaction has taken place. Although earlier crystallographic studies of antigen–antibody complexes led to the conclusion that the interacting surfaces of the two reactants are not altered by the process of binding (Mariuzza et al., 1987), subsequent crystallographic refinement showed that binding involved a certain amount of induced fit (Bhat et al., 1990; Fischmann et al., 1991; Rini et al., 1992). Recent studies of antigen–antibody interactions at the molecular level (Roberts et al., 1993) have shown that attempts to describe epitopes as static structures independently of the process of binding cannot fully account for all aspects of antigenic recognition. Confusion between static structure and dynamic processes is prevalent not only in discussions of immunological interactions, but also occurs in other areas of biochemistry (Wetlaufer, 1990).

Although it is currently fashionable to emphasize the important contributions of structural analysis to our understanding of biological phenomena, it is not possible to describe the concept of antigenic specificity only in structural terms, without incorporating the fourth dimension of time inherent in all binding activity measurements (Van Regenmortel, 1989; Greenspan, 1992). Structure is the result of selective attention to the visual representation of an object at a specific time. The structure of an epitope is thus only a visual time slice of a dynamic process of interaction.

The mutual relational dependence of antigens and antibodies implies that proteins owe their status of antigens to the existence of an immune system capable of recognizing a multitude of different structures. This recognition function is carried out by the antibody-combining sites or paratopes of immunoglobulins. Over a billion years of vertebrate evolution, paratopes have developed into highly discriminating sensing devices capable of recognizing subtle differences between molecules (see Chaps. 1 and 5). A single mouse is able to generate millions of different paratopes, each one corresponding to a unique set of hypervariable loops or complementarity-determining regions (CDR) (see Chap. 1). The adaptation of paratopes to their binding function has been achieved by the accumulation in CDRs of a much greater frequency of tyrosine and tryptophan residues than is usual at the surface of protein molecules. These aromatic side chains can make large rotations with little entropic cost, and they contribute significantly to the binding energy (Mian et al., 1991). In three lysozyme–antibody complexes studied by x-ray crystallogra-

phy, it was found that tyrosine and tryptophan residues contributed 155 of the 302 interatomic contacts between these antibodies and the antigen (Padlan, 1990).

In addition to a purely structural explanation for the extensive binding capacity of paratopes, the ultimate cause of the efficiency of antibody binding lies in the evolutionary history of the immune system in vertebrates and in the genetic mechanism of affinity maturation. Any structure or function in organisms can be fully understood only in terms of its evolutionary history. Viewed in this light, paratopes are functional entities derived from the integrated activities of the immune system.

II. TYPES OF EPITOPES

The antigenic reactivity of a protein is located in those regions of the molecule known as antigenic determinants or epitopes that are recognized by the paratopes of certain antibodies. The phenomenon of mutual recognition involves a dynamic process during which significant movements of parts of the CDRs and of the epitope take place. These movements may amount to more than 1 Å for the Cα atoms and to several angstroms for side chain atoms (Stanfield et al., 1990; Rini et al., 1992). During the interaction, certain water molecules linked to each of the two interacting species are displaced while other water molecules take on positions that make them act as a bridge or a glue between paratope and epitope (Fischmann et al., 1991). The occurrence of induced complementarity in the two interacting molecules argues against the classic interpretation of antigen–antibody interaction in terms of lock-and-key recognition (Mariuzza et al., 1987).

Epitopes are usually classified as either continuous or discontinuous (Fig. 1). This classification has mostly replaced the earlier distinction between segmented (or linear) and conformational epitopes (Sela, 1969). Conformational epitopes were defined as peptides that in solution adopt a conformation similar to that present in the corresponding region in the native protein. Antibodies to conformational epitopes were considered to be specific for this conformation and to be unable to recognize the corresponding unfolded peptide. On the other hand, antibodies to sequential epitopes were expected to bind to unfolded peptides that had not retained the original conformation present in the native protein. Because antibodies that bind to a sequential epitope nevertheless recognize a certain conformation in a peptide, usually one that is different from the native protein conformation, it would be wrong to equate sequential epitopes with conformation-independent epitopes.

The label *continuous epitope* is given to any short, linear peptide fragment of the antigen that is able to bind to antibodies raised against the intact protein. Because the peptide fragment usually does not retain the conformation present in the folded protein and mostly represents only a portion of a more complex epitope, it tends to react only weakly with the antiprotein antibodies.

The second type of epitope, known as *discontinuous epitope*, is believed to correspond to the vast majority of epitopes present in proteins. Discontinuous epitopes are made up of residues that are not contiguous in the sequence, but are brought together by the folding of the polypeptide chain (see Fig. 1). When the protein is fragmented into a number of peptides, the residues originating from the distant parts of the sequence that made up the discontinuous epitope are scattered, and each constitutive component usually is no longer recognized by the antibody. As a rule, antibodies to discontinuous epitopes will recognize the antigen only if the protein molecule is intact and its native conformation is preserved. There are exceptions, however, and it has been estimated that about 10% of the monoclonal antibodies that recognize discontinuous epitopes are also able to react with linear peptide fragments of the protein.

Instead of referring to continuous and discontinuous epitopes (Atassi and Smith, 1978), some authors speak of contiguous and discontiguous epitopes. These labels are less satisfactory, since it

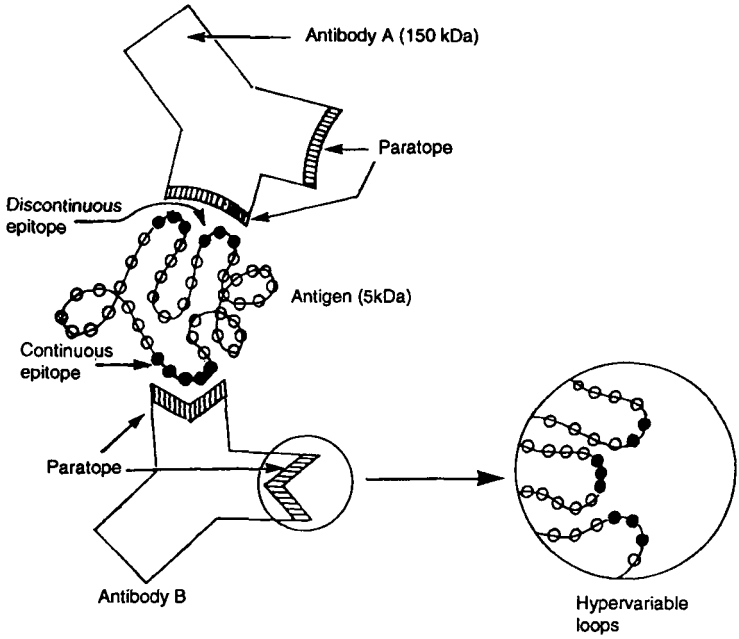

Figure 1 Interaction of two antibodies with a continuous and a discontinuous epitope of an antigen. Interacting amino acid residues are indicated in black.

is the individual constitutive residues that can be said to be contiguous, rather the epitope as a whole. Speaking of two contiguous epitopes conveys the meaning that the two epitopes occupy neighboring sites at the surface of the protein.

Some authors have challenged the view that truly native proteins possess continuous epitopes recognized by antiprotein antibodies. Laver et al. (1990) suggested that all so-called continuous epitopes represent *unfoldons* (i.e., unfolded regions of the protein antigen that cross-react only with antibodies specific for the denatured protein). Such antibodies may be present in antiprotein sera because some of the molecules used for immunization were denatured either before or after injection of the animal (Scibienski, 1973; Lando and Reichlin, 1982; Jemmerson, 1987). In reciprocal assays that use antibodies raised to peptides, it is also possible that the antibodies recognize the protein because the antigen preparation used in the assay contains some denatured molecules. The interpretation that all cross-reactions between peptides and proteins are due to antibodies to unfoldons finds some support in the fact that these cross-reactivities are often detected in solid-phase immunoassays in which the immobilized protein antigen is at least partly denatured (Kennel, 1982; Friguet et al., 1984; Spangler, 1991). On the other hand, a compelling argument against the generality of the unfoldon interpretation is found in the many studies that demonstrated that antisera raised to peptides are able to neutralize the biological activity associated with the native state of proteins. For instance, antibodies raised against certain viral peptides are able to neutralize virus infectivity (Anderer and Schlumberger, 1965; Bittle et al., 1982; Emini et al., 1985; Smyth et al., 1990). Such findings imply that the antipeptide antibodies

recognize the native state of the viral protein present in infectious virus particles. However, such instances of genuine cross-reactivities between peptide and protein do not mean that the peptide reproduces exactly the structure of the epitope present in the infectious virus particle. All that is needed is a sufficient degree of epitopic resemblance to permit antibody cross-reactivity. Although it may be only a small fraction of the total immune response against a peptide that shows this cross-reactivity, such antibodies are important in the fields of synthetic vaccines (Arnon and Van Regenmortel, 1992) and reagent preparation (Van Regenmortel et al., 1988; Boersma et al., 1993).

Concerning the number of epitopes likely to be found on a protein molecule, it is now generally accepted that the entire surface of the protein harbors numerous overlapping epitopes (Benjamin et al., 1984). This viewpoint, which arose from the results of epitope mapping using monoclonal antibodies, has superseded the earlier notion that proteins possess only a few immunodominant epitopes with discrete boundaries. The earlier view was essentially derived from the work of Atassi and collaborators, who had developed a detailed picture of the antigenic structure of myoglobin and lysozyme based on studies with polyclonal antibodies (Atassi, 1975; Atassi and Lee, 1978). These authors concluded that the total antigenicity of myoglobin resided in five continuous epitopes of about seven residues, whereas for lysozyme the antigenicity was confined to three discontinuous epitopes. These findings were challenged by subsequent studies that showed that several monoclonal antibodies (MAbs) raised against native myoglobin did not react with large peptide fragments representing the entire myoglobin sequence (Berzofsky et al., 1982). It was also shown by absorption experiments that these fragments could remove only some of the antibodies from a myoglobin antiserum (Lando et al., 1982), a finding that implied the existence of additional epitopes to those described by Atassi (1975). The conclusion that lysozyme possesses only three discontinuous epitopes (Atassi and Lee, 1978) could not be confirmed in subsequent studies by Smith-Gill et al. (1982; 1984) and was also shown to be inconsistent with data obtained with natural and synthetic lysozyme fragments (Van Regenmortel, 1988, p. 20). The view expounded by Atassi (1984) that proteins possess only a few epitopes was based on the belief that monoclonal antibodies (MAbs) give a distorted picture of the number of epitopes, because they emphasize minor epitopes that are not detectable with polyclonal antisera. Such a viewpoint is no longer tenable, since there is evidence that the range of specificities observed with a panel of MAbs is very similar to that in a polyclonal antiserum raised against the same antigen (Quesniaux et al., 1990).

In view of the relational nature of epitopes, it is possible to estimate the number of epitopes in a protein from the total number of different MAbs that can be raised against the protein. On this basis, it has been estimated, for instance, that the small insulin molecule possesses at least 115 epitopes (Schroer et al., 1983). It is interesting that the relational nexus between epitope and paratope leads to a definition of antigenic diversity in terms of the size of the immunological repertoire of the immunized host. In effect, analysis of the antigen is replaced by an analysis of the immunological tool used in the study.

III. METHODS USED FOR LOCALIZING EPITOPES

The various approaches that have been used to identify epitopes in proteins are listed in Table 1. It should be stated at the onset that the different methods of analysis lead to very different perceptions of the nature of the epitopes present in a particular protein.

Since all binding measurements are necessarily operational, the same residues of an antigen will not always be scored as participating in different types of binding assays. For instance, the result will depend on the type of probe used (i.e., free peptide versus conjugated peptide, or short peptide versus a more constrained longer peptide; Muller et al., 1986), as well as on the

Table 1 Methods Used for Localizing Epitopes in Proteins

Method	Number of residues in epitope	Criterion for residue allocation
Crystallographic analysis of antigen–antibody complexes	15–22	Contact in epitope–paratope interface
Synthetic peptides as antigenic probes in binding studies Free peptides Peptides adsorbed to solid-phase Peptides conjugated to a carrier Peptides attached to support used for synthesis	5–8	Cross-reactive binding with antiprotein antibodies
Identification of critical residues in peptides by systematic replacement of residues	3–5	Decrease in cross-reactive binding
Peptide sequences inserted in recombinant proteins	5–8	Cross-reactive binding with antiprotein antibodies
Binding of antipeptide antibodies to protein	5–8	Induction of cross-reactive antibodies
Binding of antiprotein antibodies to chemically modified or mutated proteins	3–5	Decrease in binding compared with unmodified protein
Protection of antibody-bound residues of epitope to chemical attack	5–10	Chemical reactivity of individual residues
Competitive topographic mapping	Only relative position of epitope is defined	Not applicable

experimental conditions of the immunoassay used in the study (e.g., liquid versus solid-phase assay). Although such findings may seem inconsistent, they simply reflect the operational nature of binding measurements. Operational definitions are important in science because many objects of scientific study can be properly defined only by reference to the experimental conditions that allow these objects to be studied.

A. X-Ray Crystallography

1. Crystallographic Analysis of Protein–Antibody Complexes

Our knowledge of epitope structure deduced from x-ray crystallographic studies of complexes of a protein with Fab fragments has been summarized in several recent reviews (Colman, 1988; Davis et al., 1990; Padlan, 1992; Roberts et al., 1993). Six epitopes have now been analyzed by this method, three of lysozyme (Amit et al., 1986; Sheriff et al., 1987; Padlan et al., 1989), two of influenza neuraminidase (Colman et al., 1987; Tulip et al., 1989), and one of a Fab complexed to its anti-idiotypic Fab (Bentley et al., 1990). In all of these, an area of about 700–900 $Å^2$ of the protein surface, comprising between 15- and 22-amino acid residues, was identified as being in contact with residues of the antibody. All epitopes were discontinuous and were made up of residues from between two and five separate stretches of the antigen polypeptide chain. The different paratopes were made up of residues originating from four, five, or all six of the CDRs of the antibody, as well as from some invariant framework residues.

The major binding forces contributing to the energy of interaction of five of the complexes are summarized in Table 2. An electrostatic interaction is believed to contribute about 5 kcal of binding free energy, whereas a hydrogen bond contributes only 0.5–1.5 kcal (Fersht et al., 1985; Novotny et al., 1989). As far as molecular contacts are concerned, it is usually assumed that each square angstrom of buried surface corresponds to 25 cal/mol of hydrophobic stabilization. Following crystallographic refinement of the structure of the Fab D1.3–lysozyme complex, it was found that at least eight water molecules buried in the interface were in contact with both epitope and paratope residues (Fischmann et al., 1991). These water molecules filled gaps at places where the complementarity of the two surfaces was not very close. In one case, three water molecules linked in a row by hydrogen bonds formed a bridge between antibody and antigen residues. The possibility of filling gaps by the insertion of water molecules is in line with the known role of solvent molecules in protein stabilization and antigen–antibody interaction (Tanner et al, 1992), and it refutes earlier claims, based on data obtained at insufficient resolution, that water is excluded from epitope–paratope interfaces. The presence of such water molecules is likely to complicate thermodynamic interpretations of immunological recognition as well as any modeling of epitope–paratope interfaces (Walls and Sternberg, 1992).

2. Crystallographic Analysis of Peptide–Antibody Complexes

The ability of some paratopes to recognize both the native protein and a short peptide fragment of the same protein is a useful property of antibodies that has many applications in molecular biology and related disciplines (Lerner, 1984; Walter, 1986; Van Regenmortel et al., 1988). To understand the structural basis of this type of cross-reactivity, it is important to know to what extent the conformation of the peptide in the antibody–peptide complex is the same as in the corresponding region of the intact protein. So far, important data have been obtained only in two Fab–peptide systems, one corresponding to a 19-residue protein of myohemerythrin (residues 69–87) and the other a 36-residue peptide of influenza virus hemagglutinin (Wilson et al., 1991).

Fab–Myohemerythrin Peptide Complex. Myohemerythrin (Mhr), a small protein of 115 residues made up of four α-helices, has been extensively studied by immunochemical methods (Geysen et al., 1987a; Getzoff et al., 1987). Fab fragments were prepared from MAb B13-I2, which had been raised by immunization with peptide 69–87 of Mhr, conjugated to keyhole limpet hemocyanin (KLH). Epitope mapping by means of substituted peptides showed that this antibody recognized residues 69–73 in the peptide (Fieser et al., 1987). Structure determination of the Fab–peptide complex indicated that residues 69–75 of the peptide interacted with the paratope, in good agreement with binding studies (Stanfield et al., 1990). However, rather surprisingly, the

Table 2 Binding Forces Contributing to the Energy of Interaction of Six Antigen–Antibody Complexes

Antigen	Antibody	Number of van der Waals contacts[a]	Number of putative hydrogen bonds	Number of electrostatic interactions	Ref.
Lysozyme	D 1.3	75	15	0	Amit et al. (1986)
	Hy HEL5	74	10	3	Sheriff et al. (1987)
	Hy HEL10	111	14	1	Padlan et al. (1989)
Neuraminidase	NC41	108	23	1	Tulip et al. (1989)
D 1.3	E225	100	9	1	Bentley et al. (1990)

[a]These represent molecular contact points that may not involve so-called van der Waals bonds.

conformation of the peptide bound to the Fab was very different from its conformation in native Mhr. The peptide has a type II β-turn conformation (residues 71–74) when bound to the antibody, whereas it has a preponderantly helical conformation in Mhr. The MAb B13-I2 was obtained by immunization with a coupled peptide and was selected by the criterion of being able to bind the same peptide conjugate in enzyme-linked immunosorbent assays (ELISA). Although there is no information on the conformation of the region 69–75 in the conjugated peptide, this is likely to be different from the helical conformation present in the protein. It was initially claimed that MAb B13-I2 was able to react with native Mhr because the protein in solution competed equally or better than the peptide for binding of antibody B13-I2 to Mhr adsorbed to a solid-phase (Fieser et al., 1987; Stanfield et al., 1990). However, in a subsequent study, antibody B13-I2 was able to bind to Mhr immobilized on a solid-phase, but not to Mhr in a solution-phase assay (Spangler, 1991). Only a crystallographic analysis of the complex of Mhr with antibody B13-I2 could establish if this antibody is, in fact, able to recognize the helical configuration of the region 69–75 in Mhr, although the evidence so far indicates that this is unlikely. This study of Mhr illustrates the importance of carefully controlled binding assays for correctly interpreting structural data. In the future, it seems likely that nuclear magnetic resonance (NMR) studies of peptide–antibody complexes will increasingly be used to elucidate the conformation and dynamics of peptide antigens (Scherf et al., 1992; Tsang et al., 1992).

Although polyclonal antisera raised to a peptide often contain subpopulations of antibodies that react with the cognate native antigen, these cross-reactive antibodies will not necessarily be selected during a hybridoma fusion experiment, especially if the antigen has lost its native conformation; for instance, after adsorption to a solid-phase (Darst et al., 1988; Van Regenmortel, 1989; Laver et al., 1990; Spangler, 1991). It is indeed not easy to establish unambiguously by a binding assay that an antipeptide MAb recognizes the native conformation of a protein. However, if the MAb is able to neutralize a biological activity, such as the infectivity associated with a viral antigen,it can be presumed that the antibody binds to the native structure. Such an antibody would then be a good candidate for investigating the structural basis of the cross-reactivity between protein and peptide. As far as inferring the mechanism of the cross-reaction is concerned, structural data may be compatible with more than one mechanism. For instance, elucidation of the structure of a peptide–antibody complex may indicate that a certain peptide has adopted a nativelike conformation when bound to an antiprotein antibody. However, as pointed out by Crumpton (1986), this structural information does not establish if the observed complex resulted from the antibody having selected one conformation from a mixture of different conformations, or if the recognition process involved an induced complementarity subsequent to the initial peptide–antibody interaction.

Fab–Hemagglutinin Peptide Complex. The hemagglutinin (HA) of influenza virus was the first protein of an animal virus envelope to have its three-dimensional structure elucidated (Wilson et al., 1981). A panel of MAbs was raised against the region 75–110 of HA, and the Fab fragment of one of them (MAb 17/9) was crystallized in both its unliganded form and in complex with a nonapeptide corresponding to residues 100–108 of HA (Schulze-Gahmen et al., 1988). Comparison of the two Fab structures showed that a major rearrangement in the H3 CDR loop occurred following antigen binding (Rini et al., 1992). This rearrangement consisted in a twisting of the two strands of the loop and was the result of accommodating residue Tyr-105 of the peptide. The difference observed in the free and bound conformational forms of the Fab 17/9 represents the first irrefutable demonstration that induced fit contributes to antigen–antibody recognition. Comparison of the structure of the nonapeptide bound to the Fab and its cognate structure in HA showed a major difference in the ϕ angle of the residue Pro-103, which led to a pronounced change in the bend of the type I β-turn conformation of the peptide. As a result, only a subset of the interactions seen in the Fab–peptide complex would be able to occur when the Fab binds

to HA. This partly explains the observation that HA is bound by Fab 17/9 with an affinity three orders of magnitude less than that of the nonamer peptide (Rini et al., 1992).

B. Binding Studies with Peptide Fragments

The most commonly used method for localizing protein epitopes consists of identifying which peptide fragments of the antigen are able to cross-react with antibodies raised against the intact protein. Any linear peptide that is able to bind to the protein antibodies is said to contain a continuous epitope. When peptides of decreasing size are tested, the smallest peptide that retains a measurable level of antigenic reactivity usually corresponds to a minimal epitope of about five residues (Benjamini, 1977). The degree of antigenic cross-reactivity observed with short peptides is mostly very low, with the exception of peptides corresponding to chain termini. The strong antigenicity of terminal segments of proteins is because these regions are frequently surface-oriented (Thornton and Sibanda, 1983) and more mobile than internal sections of the polypeptide chain (Westhof et al., 1984; Tainer et al., 1985). For the tobacco mosaic virus protein, even a single, highly accessible COOH-terminal residue could be recognized with a reasonable degree of specificity by antibodies (Anderer and Schlumberger, 1966). Increasing the length of peptides above five or six residues does not necessarily lead to a higher level of cross-reactivity, since longer peptides may adopt a conformation different from that present in the cognate protein (Wilson et al., 1984). It has been reported, for instance, that an antiserum to histone H2A, which reacted with peptide 1–15 of H2A, was unable to bind to histone fragments 1–39, 1–56, and 1–71 (Muller et al., 1986). It is possible that shorter peptides fold more easily into the proper conformation required for binding to the antibody (Hodges et al., 1988).

1. Synthetic Peptides as Antigenic Probes

As a result of the efficiency and convenience of solid-phase peptide synthesis (Kent and Clark-Lewis, 1985; Houghten, 1985; Geysen et al., 1987b; Plaué and Briand, 1988; Atherton and Sheppard, 1989; Plaué et al., 1990), synthetic peptides have virtually replaced natural peptide fragments as antigenic probes for epitope mapping. Several chemical strategies have been developed to increase the level of conformational mimicry between peptide and intact protein. When the peptide corresponds to a loop structure in the protein, cyclization of the peptide sometimes leads to improved antigenic cross-reactivity (Arnon et al., 1971; Fourquet et al., 1988; Jemmerson and Hutchinson, 1990; Muller et al., 1990). However, cyclization per se may not be sufficient, and a very high degree of conformational mimicry may be necessary to achieve improved reactivity (Plaué, 1990; Joisson et al., 1993). Peptides corresponding to α-helical or β-sheet structures in the protein can also be stabilized in the correct conformation by a variety of approaches (Gras-Masse et al., 1988; Satterthwait et al., 1989; Vuilleumier and Mutter, 1992). Many immunoassay formats can be used to measure the antigenic reactivity of peptides (Van Regenmortel, 1988). Since the conformation of a peptide may be modified by the assay conditions, one expects that the apparent antigenicity of a peptide will depend on the type of assay used (Muller et al., 1986).

Free Peptides. Occasionally, the free peptide in solution, when tested for its capacity to inhibit the reaction between antibody and intact protein, is more active than the coupled or immobilized peptide (Altschuh and Van Regenmortel, 1982). A recent modification of the pepscan method of peptide synthesis developed by Geysen et al. (1984) allows the peptides to be cleaved from the pins used for synthesis, and this permits the rapid testing of hundreds of peptides (Maeji et al., 1991). A combinatorial approach of peptide synthesis has recently been described that makes it possible to produce very rapidly as many as 5×10^7 hexapeptides (Houghten et al., 1991; 1992).

Peptides Adsorbed to a Solid-Phase. Peptides adsorbed to a solid-phase tend to have a significant proportion of their surface unavailable for binding to antibody, and this may reduce their antigenic reactivity, compared with free peptides in solution. To optimize the adsorption of peptides to the solid-phase, it may be necessary to test a variety of buffers (Geerligs et al., 1988) and to prevent the peptide solution from drying up during the test (Norrby et al., 1987).

Peptides Conjugated to a Carrier. It is frequently observed that the antigenic reactivity of a peptide is higher when the peptide is conjugated to a carrier protein, presumably because the microenvironment at the surface of the carrier induces the peptide to adopt a more suitable conformation for antibody recognition (Al Moudallal et al., 1985). Another reason for the better reactivity of a peptide–carrier conjugate, compared with an unconjugated peptide adsorbed to plastic, may be lower steric hindrance for antibody binding. The procedure used for conjugating the peptide may also influence its reactivity (Dyrberg and Oldstone, 1986; Schaaper et al., 1989). For instance peptide 1–8 of histone H4 was active when it was coupled to a carrier with bis-diazotized benzidine by an additional tyrosine residue at the COOH-terminal end, although the same peptide was inactive when coupled by means of glutaraldehyde (Briand et al., 1985).

Peptides Attached to the Support Used for Synthesis. Peptides can be tested for antigenic reactivity without prior cleavage from the support used during synthesis (Shi et al., 1984; Chong et al., 1992). A highly efficient method for testing large numbers of peptides is the so-called pepscan technique (Geysen et al., 1984; 1987b). In this technique, hundreds of peptides are synthesized concurrently on polyethylene pins that are assembled into a polyethylene holder with the format and spacing of a microtiter plate. This allows the peptides to be tested by ELISA while they remain attached to the pins. After each test the pins can be freed of bound antibody by sonication and retested with different antibody preparations at least 30 times. Peptides of six to ten residues can be analyzed in this manner and, usually, the set of all possible overlapping peptides of a protein, starting from the NH_2-terminus down to the COOH-terminus, are systematically screened for antigenic reactivity.

The high concentration of peptide on the pins favors bivalent binding and slow dissociation of antibody, and this facilitates the detection of very low levels of cross-reactivity with antiprotein antibodies. As a result, nonspecific reactions are sometimes observed (Savoca et al., 1991), especially when antisera are diluted less than 1:1000. In addition, unexpected cross-reactions arising from identical short sequences of two or three residues may also be detected (Geysen et al., 1986; Trifilieff et al., 1991). The specificity of pepscan reactions can be tested by analyzing the ability of free peptides, or the cognate protein, to inhibit the reaction between antibodies and pin-bound peptides.

Identification of Critical Residues in Peptides by Replacement Studies. The pepscan technique is particularly suited for analyzing the contribution of individual amino acids to the antigenic activity of a peptide. This is done by synthesizing peptide replacement sets in which each residue of the peptide is, in turn, replaced by the other 19 possible amino acids (Geysen et al., 1988). When all the analogues are tested, some critical residues are found to be essential for binding because they cannot be replaced by any residue, without impairing antigenic reactivity. Other residues can be replaced by any of the amino acids, without affecting binding (Geysen, 1985). This general replaceability may be because only main-chain atoms of the residue interact with the antibody, or that the residue acts only as a scaffold necessary to bring neighboring critical residues in the appropriate position for interaction. In the latter event, the replaceable residue is not a contact residue interacting with the paratope, and the linear peptide is not truly a continuous epitope, but is functionally discontinuous.

If the peptide does not conform to the structure of the folded protein antigen, the use of replacement sets of a short peptide for inferring which side chains are important for binding in the

corresponding epitope of the native protein may be unreliable. It is possible, for instance, that if the binding constant of the antibody for the peptide is much lower than that for the protein, certain substitutions in the peptide could simply reduce binding below the measurable threshold, whereas this would not be so with the native protein (Van Regenmortel, 1989). In a recent study, this problem was addressed by investigating whether residues that were identified as antigenically critical by the peptide-based approach were also critical within the context of the folded protein (Alexander et al., 1992). By using an expression system for recombinant myohemerythrin, a set of protein analogues containing substitutions in the region 79–84 of the native protein were analyzed. It was found that under replacement conditions of adequate stringency, the peptide-based assignment of critical residues (Getzoff et al., 1987) was confirmed by the mutagenesis results. These experiments also confirmed that a functionally active antigenic surface can be different from the corresponding solvent-exposed protein surface before binding, mainly because certain buried residues may become involved in the interaction through a process of induced fit (Alexander et al., 1992).

2. Peptide Sequences Inserted in Recombinant Proteins

Advances in genetic engineering make it relatively easy to insert a foreign peptide sequence within a vector protein (true insertion) or, alternatively, to fuse the peptide sequence at the NH_2- or COOH-terminus of the vector (terminal fusion). Recombinant genes are easily expressed in bacterial, insect, or mammalian cells, from which the recombinant protein can be extracted and purified. Since the foreign peptide is introduced in the recombinant protein by genetic association, the construct is a highly reproducible entity. In contrast, when peptide moieties are coupled to a carrier by chemical means, they encounter a variety of microenvironments at the carrier surface that may give them different conformations and antigenic reactivities. Compared with peptides obtained by chemical synthesis, peptides inserted in recombinant proteins also possess other advantages. The cost of production is low, and they can be delivered to the immune system as replicating antigens incorporated in bacterial or viral vehicles.

Methods used for expressing foreign antigens as recombinant proteins have been reviewed recently by Hofnung and Charbit (1993). Various expression systems can be used. Some bacterial vector proteins such as β-galactosidase (Broekhuisen et al., 1987) or the δ gt11 bacteriophage system (Mehra et al., 1986) are expressed only in the cytoplasm, whereas others, such as maltose-binding protein, are secreted through the cytoplasmic membrane to the periplasm or are secreted into the medium. It is also possible to express foreign peptides at the surface of bacteria using proteins such as LamB of *Escherichia coli* (Hofnung, 1991). Short peptide sequences of poliovirus (van der Werf et al., 1990) and hepatitis virus B (Charbit et al., 1987) expressed within the LamB protein were, after immunization of animals with live bacteria, able to induce antibodies that reacted with virus particles.

A variety of proteins of different origins, such as the core antigen of hepatitis B virus or the polymerase of phage MS2, have also been used as vector proteins in *E. coli* (Nicosia et al., 1987; Francis et al., 1990). Another approach is to use viral vehicles for expressing the foreign peptide. The passenger peptide is inserted into a viral coat protein, and the recombinant protein is expressed as part of the virus particle (Burke et al., 1989; Delpeyroux et al., 1990). The mapping of viral epitopes by means of expression vectors has been reviewed by Lenstra et al. (1990).

Recently, new methods have been developed that make it possible to express vast libraries of peptides on the surface of filamentous phage particles (Cesareni, 1992; Scott, 1992). Random oligonucleotides are inserted into phages (one oligonucleotide per phage), and the resultant peptide is then expressed within the filament protein on the phage surface. Phages that express a peptide epitope are screened with the antibody of interest (Christian et al., 1992), the DNA from the selected phage is amplified by polymerase chain reaction (PCR), and the peptide sequence is

determined. The peptide can be fused to the NH_2-terminus of protein pIII of which there are about five copies at one extremity of the phage (Scott and Smith, 1990; Cwirla et al., 1990; Devlin et al., 1990), or to the NH_2-terminus of protein pVIII, which is present in about 2700 copies along the phage particle (Greenwood et al., 1991). A library of 2×10^8 independent phage clones, each expressing a random hexapeptide fused to protein pIII, was used to map an epitope in the tumor antigen p53 (Stephen and Lane, 1992). This library represents 69% of the 6.4×10^7 possible hexapeptide sequences.

C. Use of Antipeptide Antibodies

The approach using antipeptide antibodies consists in immunizing animals with synthetic peptides and testing the resulting antipeptide antibodies for their capacity to cross-react with the intact protein. A positive cross-reaction is taken as evidence that the peptide approximates to an epitope of the protein.

Although it has been claimed in the past that immunization with most peptide fragments of a protein leads to a high frequency of induction of antibodies capable of recognizing the *native* protein (Lerner, 1984), this is no longer believed to be true. It seems that these claims arose because it was not realized that the antipeptide antibodies actually reacted with protein molecules that had become denatured by adsorption to plastic during the solid-phase immunoassay (Soderquist and Walton, 1980; Kennel, 1982; Jemmerson, 1987; Darst et al., 1988; Spangler, 1991).

The contention that antibodies against a highly disordered state (the peptide) are usually able to recognize the highly ordered state (the native protein), whereas the reverse is not necessarily true, has been called the order–disorder paradox (Dyson et al., 1988). It was suggested as an explanation that the peptide takes on a preferred conformation in solution that becomes stabilized either at the surface of the carrier protein or when the peptide binds to the B-cell receptor during immune stimulation. However, it is not clear why a similar induction of the correct peptide conformation could not occur when the peptide interacts with protein antibodies. On the other hand, the paradox simply vanishes when it is realized that the extent of cross-reactivity between peptides and native protein is always rather limited, irrespective of whether antiprotein or antipeptide antibodies are tested (Jemmerson and Blankenfeld, 1989).

To have a good probability that antibodies raised to a peptide will recognize the cognate protein in its *native* state, the peptide should correspond to a continuous epitope of the protein. This requirement of the peptide to be used for immunization has led to many attempts to predict the location of continuous epitopes in proteins from certain features of their amino acid sequences (Hopp, 1986; Parker et al., 1986; Pellequer et al., 1991). Numerous studies have shown that the location of continuous epitopes in proteins is correlated with structural parameters, such as accessibility, hydrophilicity, segmental mobility, and sequence variability. However, different workers disagree about which parameter correlates best with antigenicity (Tainer et al., 1985; Novotny et al., 1987; Van Regenmortel, 1989). Recently, the value of 28 propensity scales used for predicting antigenicity has been analyzed, using 14 proteins containing 85 identified epitopes (Pellequer et al., 1994). The method of analysis calculated how many residues of each protein were correctly or wrongly predicted to be antigenic. It was found that hydrophilicity scales gave 53–61% correct predictions, whereas accessibility and flexibility scales gave 51–60% and 50–55% correct predictions, respectively. The highest level of correct prediction (70%) was reached with a turn prediction method based on the location of four-position turns. These fairly low levels of correct prediction may be partly due to the presence, in most continuous epitopes, of indifferent replaceable residues not directly implicated in the binding interaction.

Methods used to produce antipeptide antisera have been described in several reviews (Walter, 1986; Muller, 1988; Boersma et al., 1993). For peptides shorter than about 15 residues, it is

customary to use peptide–carrier conjugates for immunization (Briand et al., 1985; Soutar and Palfreyman, 1986; Smith et al., 1993). Since it is impossible to predict which method of conjugation will give the best results (Dyrberg and Oldstone, 1986; Bahraoui et al., 1987; Mariani et al., 1987; Muller, 1988; Schaaper et al., 1989; Boersma et al., 1993), it is advisable to use more than one coupling procedure and immunization schedule. Branched peptides, known as the multiple antigen peptide (MAP), were developed by Tam (1988) to enhance the immunogenicity of peptides. The MAPs, which consist of a polylysine core onto which the peptide of interest in synthesized, avoid the formation of antibodies to a carrier protein (Tam, 1988; McLean et al., 1991) and are particularly effective when used as antigenic probes in solid-phase assays (Marsden et al., 1992). However, MAPs do not always lead to the formation of antibodies cross-reactive with the intact protein (Briand et al., 1992).

In addition to pointing to the presence of continuous epitopes in proteins, antipeptide antibodies have many applications in diagnostic immunoassays (Joisson et al., 1992; Maruyama et al., 1992; Sohma et al., 1992), in the immunoaffinity purification of proteins (Wojchowski et al., 1987), and for fusion proteins with peptide tails (Hopp et al., 1988). Antipeptide antibodies can be affinity purified by means of polystyrene beads coated with peptides (Karlsen et al., 1990).

D. Binding Studies with Chemically Modified or Mutated Proteins

In this method, monoclonal antibodies are used to measure the extent of antigenic cross-reactivity between related proteins presenting known amino acid substitutions or chemical modifications (Van Regenmortel, 1984). If the substitution leads to a change in antibody binding, it is assumed that the altered residue is involved in the structure of an epitope (Hornbeck and Wilson, 1984). Although single substitutions at the surface of a protein tend to cause only a local change, with no long-range structural alterations (Benjamin et al., 1984), it may happen that certain mutations alter the antigenicity indirectly through a distal conformational change (Al Moudallal et al., 1982; Blondel et al., 1986; Collawn et al., 1988). Changes in paratope specificity have also been observed after the introduction of substitutions outside of the CDR residues directly in contact with the antigen (Chien et al., 1989; Lavoie et al., 1992). Initially, studies with mutants included proteins, such as lysozyme and cytochrome *c*, for which there are large numbers of homologous, evolutionarily related variants. In recent years, variants generated by site-directed chemical modifications (Cooper et al., 1987; Oertle et al., 1989) or by site-directed mutagenesis (Smith and Benjamin, 1991; Smith et al., 1991) have been increasingly studied. Alanine-scanning and homologue-scanning mutagenesis have been used successfully to map a variety of epitopes (Cunningham and Wells, 1989; Wells, 1991; Jin et al,. 1992). Mutations to alanine are used because it is the mildest means of removing an interaction beyond the β-carbon, without imposing disruptive or productive interactions.

Chemical modifications at 23 residue locations in the sequence of myoglobin were analyzed by Atassi and collaborators in their classic studies of the antigenic structure of myoglobin by means of polyclonal antibodies (Atassi, 1975; 1977). Epitopes in snake toxins have been located by measuring the change in affinity of antitoxin monoclonal antibodies for chemical derivatives of the toxin modified at single residue locations (Boulain et al., 1982; Menez et al., 1992).

E. Differential Sensitivity of Free and Antibody-Bound Epitopes to Chemical Modifications, Proteolysis, or Amide Exchange

Binding of an MAb to its antigen will prevent certain contact residues of the epitope from reacting with a particular chemical or an enzyme. The differential rate of proteolysis of peptide bonds

within an epitope when it is free or bound to an MAb has been used to characterize two discontinuous epitopes of cytochrome *c* (Jemmerson and Paterson, 1986). By comparing the degree of protection from reaction with acetic anhydride of 18 lysine and 7 threonine side chains in free and antibody-bound cytochrome *c*, it was possible to assign certain residues to a discontinuous epitope of the protein (Burnens et al., 1987). Subsequently, the same technique was used to further characterize four discontinuous epitopes of the same protein (Oertle et al., 1989).

The ability of deuterium to exchange with amide protons in a protein is greatly decreased for the residues of an epitope that are buried in the antibody-combining site (Paterson, 1992). The effect of antibody binding on the exchange kinetics of amide hydrogens can be determined by two-dimensional NMR, and this approach has been used to identify certain residues in discontinuous epitopes of cytochrome *c* (Paterson et al., 1990). Recently, the same technique has been applied to an epitope–paratope pair for which the crystal structure of the complex had previously been determined by x-ray crystallography (i.e., the MAb HyHEL5–lysozyme complex; Benjamin et al., 1992). A total of 15 amide proteins showed altered exchange kinetics in the presence of the complex. Five of these protons were located on residues identified as belonging to the epitope by x-ray crystallography. In addition changes in amide exchange rates were observed for five residues located at the perimeter of the epitope, as well as for five residues that were remote from the epitope defined by structural analysis. These latter effects were attributed to a reduction in fluctuations of the protein, resulting from the binding interactions. Such a decrease in the local unfolding properties of the protein corresponds to a reduction in the number of accessible energy states of the protein. These findings indicate that long-range changes can occur in proteins by the formation of protein–antibody complexes and imply that the assignment of residues to an epitope on the basis of amide exchanges may be unreliable (Benjamin et al., 1992). The results of these amide exchange studies also cast doubt on some of the conclusions drawn from the structural analysis of the Hy HEL5–lysozyme complex. On the basis of the earlier crystallographic data, it was concluded that water molecules were excluded from the epitope–paratope interface (Davies et al., 1990). However, in view of the fairly low amide exchange protection factors for contact and buried residues observed by Benjamin et al. (1992), it seems that the interface is, in fact, solvent-accessible to some degree. It cannot be excluded that further refinement of the crystallographic data, as performed by Fischmann et al. (1991), will reveal the presence of water molecules in the interface. Such interstitial water may simply represent residual water of hybridation that is not removed by what is likely to be a less than perfect complementary fit between antigen and antibody.

F. Competitive Topographic Mapping

Competitive binding assays with pairs of MAbs are commonly used to determine the relative position of epitopes on the surface of a protein (Berzofsky, 1984). In such assays, two epitopes will be recognized as different only if they are far enough apart to allow simultaneous binding of the two MAbs. In many cases, however, MAbs directed against distinct, but neighboring, epitopes will be prevented from binding simultaneously to the antigen surface because of steric hindrance.

By testing all possible pairs of MAbs in double–antibody-binding assays, each antibody can be assigned to particular reaction patterns relative to other MAbs. This makes it possible to construct two-dimensional "surfacelike" maps that mimic the distribution of epitopes on the surface of the antigen. The ability of pairs of MAbs to bind concurrently to the antigen is usually measured by ELISA, and this requires labeling one of the two MAbs. Biotinylation of the antibody is preferable to enzyme conjugation (Zrein et al., 1986). In addition to ELISA, techniques such as gel filtration high-performance liquid chromatography (Mazza and Retegui, 1989), gel electrophoresis (Wilson and Smith, 1984), light-scattering spectroscopy (Yarmush

et al., 1987), and surface plasmon resonance using a biosensor instrument (Fägerstam et al., 1990) have been used for topographic epitope mapping. The automated biosensor technique (BIAcore, Pharmacia, Uppsala), which is described in detail in Chapter 36 is particularly suited for this type of epitope mapping (Dubs et al., 1992).

IV. CONCLUSIONS

In this chapter, the recognition of antigens by the immune system was described in terms of two relational concepts, epitope and paratope, and the same mechanisms were shown to be at work in the capacity of antibodies to recognize either native proteins or short peptides. The CDRs of immunoglobulins represent a functional adaptation that endows these proteins with an immense discriminatory power and allows them to interact with myriads of complementary entities called epitopes. A more restricted use of the term epitope has been proposed by Laver et al. (1990). These authors suggested that the term *epitope* should be reserved for those structures on *native* proteins that bind antibodies, whereas antigenic structures present on unfolded or denatured proteins or in peptides should be called something else, for instance, unfoldons. This somewhat impractical proposal probably arose from the realization that considerable confusion had been created in the past by the failure of many authors to distinguish between the epitopes of native and denatured proteins. Unfortunately, it is not always straightforward, except in extreme cases, to assess to what extent a protein in an immunoassay is native or partly denatured. Furthermore, there are examples for which the antibody is able to bind both the native and denatured forms of the protein. Another restriction to the use of the term epitope was proposed by Atassi (1984), who suggested that epitopes should not be defined in terms of only their ability to bind to paratopes, but that they should also be shown to be able to induce an immune response in a competent host. Since immunogenicity cannot be defined outside of the host context, and since it is impossible to monitor conformational changes occurring in the antigen after it has been injected into an animal (the black-box conundrum), it seems more practical to label as epitope any entity able to interact in vitro with the paratope of an immunoglobulin molecule.

The relation between an antibody and its antigen is never of a completely exclusive nature. Although it has been accepted for many years that an individual epitope can be recognized by several different paratopes, the reverse situation—namely, that antibodies are polyspecific— has been accepted much more slowly. In addition to recognizing the epitope against which it was elicited, a paratope will also bind to a variety of related epitopes that share some structural features with the epitope used for immunization (Roberts et al., 1993). It is common for an antibody to bind to its homologous antigen with higher affinity than to heterologous antigens, although the reverse situation is not at all rare. Heterospecific or heteroclitic binding occurs when the antibody reacts more strongly with other antigens than with the one against which it was raised. It seems that heterospecific binding is found whenever it is looked for (i.e., when the antibody is tested against a series of closely related analogues of the immunogen; Al Moudallal et al., 1982; Underwood, 1985; Harper et al., 1987), and its occurrence can be of considerable help when attempts are made to produce monoclonal antibody reagents specific for the individual members of a family of related proteins (Frison and Stace-Smith, 1992). Heterospecificity is due to the fact that the clonal selection of a B cell, which eventually leads to antibody production, can be triggered by an immunogen endowed with only moderate affinity for the B-cell receptor. Since a very high degree of fit between epitope and paratope is not required for initiating B-cell differentiation, there is nothing unusual in the finding that antibodies often show a higher affinity for related epitopes endowed with a superior degree of complementarity with the paratope.

In view of the widespread occurrence of cross-reactive fit between paratopes and heterologous epitopes, there is no easy way to define the specificity of an antigen–antibody interaction.

Perfect fit between epitope and paratope is not a meaningful concept, because it would imply that heterospecific binding and further affinity maturation are ruled out. The degree of specificity of an interaction cannot be linked directly to the size of the antibody affinity constant, and it is generally more meaningful to compare specific interactions in terms of their discrimination potential. It is the wish of the investigator to differentiate between two entities that provides the necessary criterion for deciding whether a particular antigen–antibody reaction is specific or not. Specific reagents are those that achieve the level of discrimination that is required in any particular case. The same antibody may thus be called specific or nonspecific, depending on what the investigator is trying to achieve.

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