Antigenicity: Some Molecular Aspects

Synthetic antigens help our understanding of immunological phenomena on a molecular level.

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Immunity toward a virus, allergy due to pollen, rejection of a kidney transplant, and autoimmune diseases are different facets of the same basic immunological mechanism. The material capable of provoking an immune response is called antigen, and a common response is the production of antibodies, even though we know other types of immune reaction such as, for example, delayed hypersensitivity which is cell-mediated. In recent years we have seen not only tremendous progress in the elucidation of the structure and biosynthesis of antibodies (*1*), but we have also achieved a better understanding of antigenicity, largely because of the development and use of synthetic antigens (*2, 3*).

The term "antigenicity" has been used often to describe both the ability to elicit antibodies and the capacity to react with antibodies. Thus, some of the antibodies formed upon immunization with *Bacillus anthracis* react well with poly-γ-D-glutamic acid, a polymer isolated from the cell wall of this bacterium (*4, 5*). This antigen does not elicit antibody formation when administered to experimental animals. For the sake of clarity I distinguish between two separate notions: (i) immunogenicity, which I define as the capacity to provoke an immune response and which is independent of the specificity of the antibodies formed; and (ii) antigenic specificity, which is reflected in the nature of the antibody combining site. There is a certain formal analogy with enzymes, where we are accustomed to differentiating between catalytic sites and specificity sites. Similarly, for an antigen we could distinguish between those areas of the molecule necessary to render it immunogenic and those contributing to its antigenic specificity.

Until recently, all the antigens known were of natural origin (microbial, plant, animal). The efforts to understand better the specificity of immunological reactions led the immunochemists to modify chemically such natural antigens (*6*). I would like to call the modified materials artificial antigens. Landsteiner demonstrated the great specificity of serological reactions by using well-defined small molecules (haptens) which he attached to proteins, thus changing the antigenic specificity of these immunogens. In contrast to artificial antigens, which are immunogenic to begin with, synthetic molecules may be helpful in elucidating the reasons for immunogenicity and for specificity.

In response to an immunogenic molecule with more than one type of antigenic determinant, an animal may produce varying amounts of antibodies of different specificities. I use the term *immunopotent* for a determinant provoking the formation of specific antibodies in high concentration. Some determinants may be *immunosilent* under certain circumstances, but these may become immunopotent as a result of either chemical modification or fragmentation. In immunochemical studies a certain small portion of the antigenic determinant is of crucial importance in defining its specificity, and this portion has been termed by Heidelberger *immunodominant* (*7*). Many haptens in various artificial antigens are smaller in size than the complete determinant, and they serve only as the immunodominant part of a determinant in which the protein carrier also participates (*8*). The word "carrier" is used here in the chemical sense, and it should be distinguished from the concept of "carrier" in an immunological sense—namely that moiety of the immunogenic molecule which does not contribute at all to the specificity (*9*). The carrier, in this immunological sense, still has an effect on the structure and biosynthesis of antibodies.

Even though bacteria, viruses, or cells are often called antigens, they really are multimolecular mixtures of polydeterminant antigens. Even proteins are in most cases rather complicated antigens, with a broad mosaic of determinants.

Synthetic Antigens

The availability of synthetic poly-α-amino acids provided a new approach to evaluate physical and biological properties of proteins (*10*) and led to efforts to detect their possible antigenicity (*11*). Rather than try at random the capacity of various polymers and copolymers of amino acids to induce antibody formation, my colleagues and I have asked ourselves whether we can, by means of chemical modification, enhance the immunogenicity of a very poor immunogen. We attached peptide chains of tyrosine to gelatin and converted it from a very poor into a powerful antigen in guinea pigs and rabbits (*12*). The extent of the immunogenicity depended on the amount of tyrosine attached. As little as 2 percent tyrosine sufficed to enhance strongly the antigenicity of gelatin. The antibodies formed cross-reacted well with gelatin, so that we had the feeling that in this case tyrosine was a kind of enhancer; it makes the molecule a better immunogen without changing the specificity very much. On the other hand, if we had as much as 10 percent of tyrosine on gelatin, it was again a very good antigen, but this time the antibodies were directed to tyrosyl peptides and not to gelatin.

In view of these experiments, the question arose whether we need the gelatin at all or whether we can replace it by a synthetic macromolecule that is essentially nonantigenic. Our choice was a multichain polymer of DL-alanine. This branched polymer

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consists of polymeric side chains of DL-alanine attached to the ε-amino groups of a polylysine backbone; it is obtained in a relatively high molecular-weight range by reacting *N*-carboxy-DL-alanine anhydride with poly-L-lysine. We chose the multichain polymer of DL-alanine rather than poly-L-alanine, because the latter is utterly insoluble in water. When we attached some tyrosine, or tyrosine and glutamic acid, to this nonantigenic (*13*) material and injected the compounds obtained into rabbits, the antiserums formed contained antibodies to these materials, and these antibodies were highly specific (*14*). Thus, we had for the first time a synthetic polymer which was a potent and specific antigen (Fig. 1). Independently Gill and Doty (*15*) have shown that a linear synthetic polypeptide, composed of L-glutamic acid, L-lysine, and L-tyrosine is a strong antigen in rabbits.

The above-mentioned studies, as well as many others (*2*), proved that linear and branched synthetic polymers composed exclusively of α-amino acids bound through peptide linkages may be powerful and specific antigens in rabbits, guinea pigs, mice, rats, goats, monkeys, and humans.

The synthetic approach offers the advantage that, once the immunogenicity of one synthetic material has been unequivocally demonstrated, tens of analogs may be prepared and tested. If the chemistry of these compounds is known, it seems possible, through a study of copolymers showing only limited variations in their chemical formulas, to arrive at conclusions concerning the role of various structural features in their antigenic function. The problems to be considered should include, among others, the roles of shape, size, and composition of the macromolecule, of the locus in the molecule of the area important for immunogenicity, and of the optical configuration of its component amino acids as well. They should also include the roles of electrical charge and of the steric conformation of the macromolecule.

Homopolymers of α-amino acids are not immunogenic under the usual conditions of immunization, with the exception of poly-L-proline in guinea pigs (*16*). Antibodies with specificity directed toward homopolymers may be obtained by using the homopolymers as haptens. This may be accomplished either by chemical attachment of homopeptides to proteins (*17*), or—in the case of polyelectrolytes—by mixing them with an immunogenic polyelectrolyte of opposite charge (*18*). Thus, antibodies to polylysine and polyornithine were obtained upon injecting rabbits with a complex of these polybases with phosphorylated bovine serum albumin (*19*), whereas a complex of synthetic poly-α-L-glutamic acid, or natural poly-γ-D-glutamic acid, and methylated bovine serum albumin led to specific antibodies to the respective polyglutamyl determinants (*20, 21*). An electrostatic complex formed between poly-L-glutamic acid and poly-L-lysine appeared to have elicited antibodies specific for each individual homopolymer (*22*).

A wide variety of copolymers of amino acids are immunogenic, and the amount of antibody is enhanced when aromatic amino acids are present (*2, 23*). Similarly, attachment of peptides composed of aromatic amino acids enhanced the immunogenicity of gelatin (*12*). This is not due to the aromatic character, for attachment of cyclohexylalanine peptides converted gelatin also to a good immunogen (*24*), and the incorporation of alanine enhanced the immunogenicity of synthetic copolymers in some cases (*23*).

Interesting species differences have been observed. Thus, polymers of two different amino acids are immunogenic in rabbits and guinea pigs but not in mice and men. In all species, polymers containing three or four different amino acids have been excellent immunogens (*2, 25*). While polyamino acids of increasing complexity seem to be better immunogens, it should be kept in mind that this may reflect not only increased variations in composition, but also characteristic conformations. As discussed below, in those antigens which interest us most—like the proteins—the immunogenicity and antigenic specificity are controlled primarily by their higher order structure, rather than by amino acid composition (*26*).

In a series of polymers containing many electrical charges, those with a high net charge were very poor immunogens (*23*). On the other hand, as most natural antigens are charged, it was of interest to find out whether a molecule without charges may be immunogenic at all. We investigated the immunogenicity of a branched polymer that contained tyrosine and was both soluble in water and nonionizable in the neutral *p*H range, and we found that it was immunogenic in rabbits (*27*). Thus, the presence of electrical charges on a macromolecule is not a minimum requirement for it to be immunogenic (*27*).

The antigenic synthetic multichain polypeptide discussed earlier is shown schematically on the left side of Fig. 1. We have prepared a molecule with the same size, shape, and composition, but in this case the peptides of tyrosine and glutamic acid are attached to the polylysine, and the alanine peptides are on the outside (right side of Fig. 1). This compound is nonantigenic in rabbits. From a detailed study with multichain polymers of different average distances between the polypeptidic side chains, it was possible to conclude that, in order to elicit biosynthesis of antibody, the immunogenically important area must be readily accessible and cannot be hidden in the interior of the molecule (*14*). It is interesting that, before alanylation, the intermediate polymers in which side chains of tyrosine peptides—or of peptides of tyrosine and glutamic acid—are attached to a polylysine backbone are good antigens. Thus, by chemical modification, antigenic materials may be converted into nonantigens, whereas nonantigenic materials may become antigenic. The above-mentioned results also suggest that the synthetic polypeptide antigens investigated were not split in vivo to a significant extent before reaching the site of the biosynthesis of the antibody. Multichain polymers of different side-chain densities, as well as linear polyamino acids, may be immunogenic. Thus the overall shape of the molecule does not seem to be a critical factor in immunogenicity.

Molecular Weight of Synthetic Antigens

The question of the lower limit of molecular weight at which a substance may be immunogenic has been of great concern to immunologists. The smallest synthetic polypeptide antigens among the substances investigated until recently had average molecular weights in the range of 4000 to 5000 (*2*). However, synthetic peptides of even lower molecular weights may prove antigenic, provided that they possess the required immunogenic features. Thus, α-dinitrophenylhepta-

L-lysine (molecular weight, 1080) is immunogenic in guinea pigs, though not in rabbits, whereas α-dinitrophenylhexa-L-lysine is not immunogenic in either guinea pigs or rabbits (*28*). Replacement with D-lysine of the fifth L-lysine in α-dinitrophenylnona-L-lysine converts it into a substance completely devoid of the capacity to induce an immune response (*29*). It is not clear whether the dramatic differences are due to a threshold in binding capacity at some stage of antibody biosynthesis, or whether they are due to drastic size-dependent conformational changes (*30*).

Hexa-L-tyrosine to which one azophenylarsonate group per molecule was attached was immunogenic in guinea pigs and rabbits, while similarly substituted tri-L-tyrosine and *N*-acetyl-L-tyrosine amide (molecular weight, 450) elicited both delayed sensitivity and antibody production in guinea pigs (*31*). It is pertinent that angiotensin (molecular weight, 1031) and a bacitracin derivative to which three dinitrophenyl groups were attached (molecular weight, 1928) were also shown to be immunogenic (*32*).

The synthetic antigens may be immunogenic even at very low doses. As little as 4 micrograms of poly (Tyr,-Glu)-poly DLAla--poly Lys (*32a*) is sufficient to immunize a rabbit (*33*). In discussing immunogenicity, one must consider in addition to the chemical characteristics of the immunogen such variables as methods used for immunization, species and strain of the experimental animal, the role of adjuvant, and the physical form of the molecule administered. Insolubilization by cross-linking (*34*) or attachment to an insoluble carrier may enhance considerably immunogenicity (*35*).

Our knowledge about the number of antigenic specificity determinants that a protein contains is still limited. Upon inspecting some simple synthetic branched antigen systems in which the number of repeating determinants is very high, it became apparent that the valency of synthetic antigens does not differ significantly from the valency of natural protein antigens of a similar molecular weight (*14*). It seems thus that in the natural antigens also the number of specificity determinants may be much higher than the experimentally determined valency, and that steric factors determine the maximum number of antibody molecules which may react with one antigen molecule.

Size and Nature of Antibody Sites

The exquisite specificity of antibodies (*6*), reviewed by Goodman (*36*), is characteristic also of antibodies to synthetic antigens. The antigenic determinants are of well-defined and rather narrow serological specificity, as apparent from reactions of antibodies with various chemically related and unrelated linear and branched polypeptides, proteins, and peptidyl proteins (*2*).

In analogy to the pioneering studies of Kabat on the dimensions of the combining sites of antibodies to dextran (*37*), several studies have appeared in which antibodies directed to polypeptidyl determinants were characterized by the extent of inhibition of the antigen-antibody reaction with peptides of increasing size (*17, 19, 38*). Recently, for a more precise evaluation of the results, instead of preparing peptidyl proteins by polymerization techniques (*17*), we have used as immunogens proteins to which peptides of defined length and structure have been attached (*39, 40*). Peptides of the structure $(DAla)_n$-Gly ($n = 1$ to 4) were coupled to ribonuclease in a one-step synthesis. The resulting conjugates induced in rabbits peptide-specific antibodies, detected by their precipitin reaction with rabbit serum albumin to which the same peptides were attached. These precipitin reactions were inhibited with peptides of general structures: $(DAla)_n$ ($n =$ 2 to 5); $(DAla)_n$-Gly ($n = 1$ to 4); and $(DAla)_n$-Gly-ε-aminocaproic acid ($n =$ 1 to 3). From cross-precipitation and inhibition experiments, it was concluded that the size of the combining sites was in all cases such as to accommodate four amino acid residues, and thus the antigenic determinant is a tetrapeptide. The protein participates in the antigenic determinant only when the hapten attached is smaller than a tetrapeptide.

A convenient way to analyze the properties of different regions of the active site of enzymes is to subdivide the active site (*41*). The combining site of the antibody may be subdivided into subsites (S_1 to S_4), each accommodating one amino acid residue of the antigenic determinant. Thus, DAla-Gly-ε-aminocaproic acid inhibits the precipitin reaction between DAla-Gly-(rabbit serum albumin) and antiserum to DAla-Gly-ribonuclease much more efficiently than $(DAla)_2$-Gly-ε-aminocaproic acid. The immunodominant role of the most exposed portion of the determinant (NH_2-terminal amino acid residue) may be understood in the system described by assuming that the interaction starts from subsite S_1 of the antibody, which always interacts with the NH_2-terminus of the related peptide (Fig. 2). When the antibody interacts with the peptide DAla-Gly-ε-aminocaproic acid, a complete geometrical fit is obtained. In the case of $(DAla)_2$-Gly-ε-aminocaproic acid peptide, subsite S_1 cannot interact effectively (if at all) with the second D-alanyl residue which is lacking free α-amino group.

Immunoglobulin G antibodies differ from IgM (*42*) antibodies in their molecular size, electrical charge, and

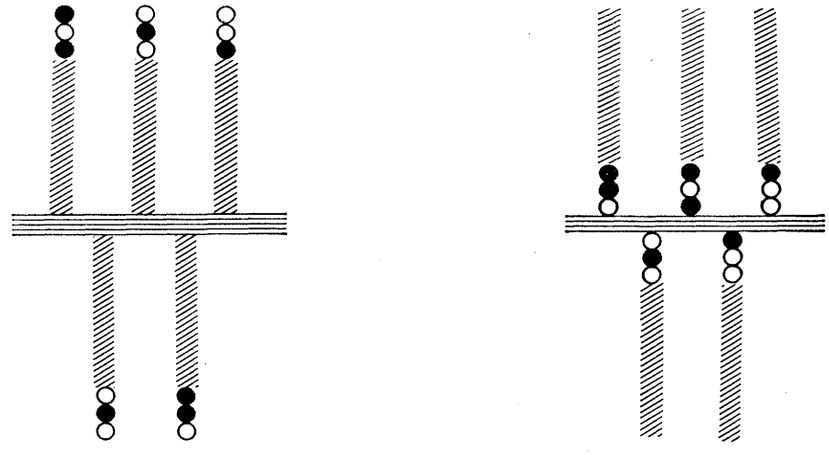

Fig. 1. A multichain copolymer in which L-tyrosine and L-glutamic acid residues are attached to multi-poly-DL-alanyl--poly-L-lysine (left); abbreviated as poly (Tyr,Glu)-poly DLAla--poly Lys), and of one in which tyrosine and glutamic acid are attached directly to the lysine backbone and then elongated with alanine peptide (right); horizontal lines, -poly-L-lysine; diagonal hatching, -poly-DL-alanine; closed circles, -L-tyrosine; and open circles, -L-glutamic acid.

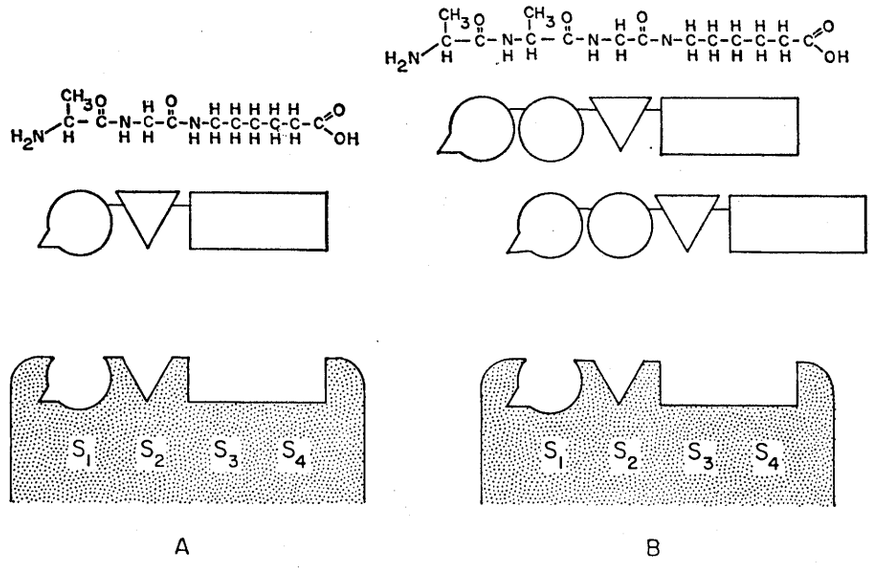

Fig. 2. The combining site of antibody to the antigenic determinant DAla-Gly-ε-aminocaproic acid. The combining site is composed of four subsites (S_1 to S_4) "designed" to accommodate the following portions of the determinant: S_1, the NH_2-terminal D-alanine containing a free α-amino group; S_2, the glycine residue; S_3 and S_4, the COOH-terminal ε-aminocaproic acid. (A) represents the lining up of the peptide DAla-Gly-ε-aminocaproic acid on the combining site. (B) represents two possible ways for lining up of $(DAla)_2$-Gly-ε-aminocaproic acid on the combining site (*39*).

antigenic properties. It was, therefore, of interest to compare the size of their combining sites. We used for this purpose the efficiency of inhibition of the antigen-antibody reaction in the poly-D-alanyl system, by means of D-alanine peptides of increasing size. We tested this both with rabbit and goat antibodies, using either inhibition of the precipitin reaction or inhibition of the inactivation of bacteriophage modified by chemical attachment of D-alanyl peptides (*43*). In all cases penta-D-alanine was not a significantly better inhibitor than tetra-D-alanine, whereas tri-D-alanine was a less efficient inhibitor. Thus, the antigen-combining sites of both IgG and IgM antibodies are similar in size, and their dimensions are such as to accommodate a tetrapeptide.

Role of Optical Configuration

Antibodies are stereospecific (*6*); indeed, there is no cross-reaction between antibodies to poly-L-alanyl and poly-D-alanyl determinants (*17*). On the other hand, peptides of D-tyrosine are as efficient as those of L-tyrosine in enhancing the immunogenicity of a molecule (*27*). What about the immunogenicity of copolymers composed exclusively of D-amino acids? Several investigators reported failure to detect any antibodies (*2, 25, 44*), with one notable exception (*45*), even though the corresponding polypeptides of L-amino acids were immunogenic. More recently, it became apparent that the immunogenicity of D-amino acid polymers in mice (*46*) and rabbits (*23, 47*) is strongly dependent on dose. For example the response in mice to the D-isomers exhibited a sharp maximum around 1 microgram per mouse, whereas that to the L-isomer was largely independent of dose. It thus seems that the inefficient formation of antibodies to polymers composed exclusively of D-amino acids is due to their slow and incomplete catabolism (*48, 49*).

The linear copolymer of D-tyrosine, D-glutamic acid, and D-alanine, which is catabolized and excreted only very slowly in mice (*49*), is immunogenic when administered to adults together with an adjuvant, but without adjuvant it very readily causes immunological tolerance (*46*). This tolerance, or paralysis, caused by indigestible D-amino acid copolymers resembles that caused by indigestible bacterial capsular polysaccharides.

We have reinvestigated the role of optical configuration in immunogenicity, by making use of a family of branched polymers derived from multichain polyproline (*50*). In multichain polyproline, linear polymeric chains of proline are attached to the ε-amino groups of polylysine. Multichain polyprolines of either optical configuration did not provoke antibody formation in rabbits. Attachment of short peptides composed of L-tyrosine and L-glutamic acid or L-phenylalanine and L-glutamic acid to multichain polyproline of either optical configuration led to materials provoking a good immune response in rabbits. Thus, the attachment of as little as 8.5 percent of L-amino acid residues on the outside of a macromolecule composed exclusively of D-amino acids converted it into a good immunogen.

In contrast, materials obtained upon attachment of short peptides of D-tyrosine and D-glutamic acids or of D-phenylalanine and D-glutamic acid to either multichain poly-D-proline or poly-L-proline did not lead to a detectable immune response in rabbits when the usual high dose was used for immunization, but they were weakly immunogenic at low dose. Thus, a macromolecule containing as much as 95.5 percent of L-proline and L-lysine, but having all its dense polymeric side chains terminated with D-amino acids, is as poor an immunogen as a similar macromolecule composed exclusively of D-amino acids. This seems due to the lack of endopeptidases capable of splitting peptide bonds between two L-proline residues and thus to incapability of the animal to catabolize a macromolecule in which every poly-L-proline chain is linked to an ε-amino group of lysine on one end and to a peptide composed of D-amino acids on the other end (*51*). The good immunogenicity of a polymer composed mostly of D-amino acids, but possessing as its specificity determinants short peptides of L-amino acids, may be interpreted on the same basis as being related to the digestion of the antigenic determinant composed of L-amino acids and thus not dependent on the slow catabolism of multichain poly-D-proline.

Role of Conformation

Spatial folding of proteins plays an important role in determining their antigenic specificity (*6, 36*), as apparent from the poor reaction, or total lack of cross-reaction, between denatured proteins and antibodies to the same proteins in their native form. Thus, for example, antibodies to native bovine pancreatic ribonuclease do not react at all with the ribonuclease oxidized by performic acid, which is a randomly coiled chain devoid of disulfide bridges; nor do antibodies to ribonuclease oxidized by performic acid react with the

native enzyme (*52*). Similarly, antibodies prepared in goats (goat antiserum) against rabbit immunoglobulin G do not react with the rabbit IgG after all its disulfide bridges are opened by reduction with 2-mercaptoethanol (*53*). This is undoubtedly due to changes within the conformation of the protein molecule, resulting in loss of the original antigenic determinants. Conformation designates here a particular arrangement of atomic positions of a molecule, which can be achieved without the reorganization of chemical bonds. An antigen can provoke antibodies against many different determinants present in its molecule, and some of these antibodies may be overlapping. Thus, antiserums against a protein antigen usually contain a population of antibodies of differing specificity, having as a common denominator the capacity to react immunospecifically with the antigen. In some cases, for example, the protein of tobacco mosaic virus, the relative immunopotency of the determinants differs depending on whether the intact virus or the isolated protein is used for immunization (*54*).

There are many reported examples in which antibodies were used for detection of different conformations in proteins. For example, metmyoglobin forms a reddish-brown precipitate with antiserum to metmyoglobin. Antiserums to apomyoglobin give white precipitates with apomyoglobin, but also with metmyoglobin (*55*). The release of ferriheme from metmyoglobin must have been due in this case to a change in the conformation of the cross-reacting antigen upon reaction with antibodies to myoglobin. Conformational changes are most probably also the cause for the marked effect of antibodies to hemoglobin on the oxygen equilibrium of human hemoglobin (*56*). Antibodies may stabilize the native conformation of a protein, and they may even prevent its denaturation by heat, as reported recently for bovine acetylcholinesterase (*57*).

I would like to call a sequential determinant one due to an amino acid sequence in a random coil form, and antibodies to such a determinant are expected to react with a peptide of identical, or similar, sequence. On the other hand, a conformational determinant results from the steric conformation of the antigenic macromolecule and leads to antibodies which would not necessarily react with peptides derived from that area of the molecule. It seems that antibodies to native proteins are directed mostly against conformational rather than sequential determinants.

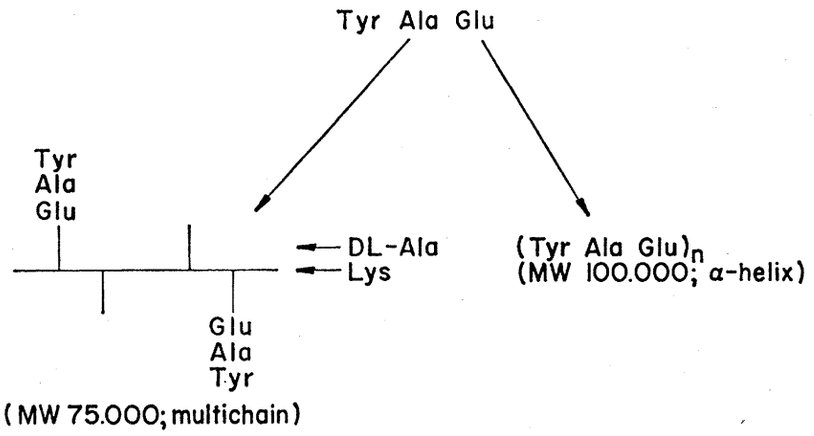

Fig. 3. Synthetic branched polymer in which peptides of sequence Tyr-Ala-Glu are attached to the amino terminals of polymeric side chains in multi-poly-DL-alanyl--poly-L-lysine (left) and of a periodic polymer of the tripeptide Tyr-Ala-Glu (right).

In order to elucidate the role of the conformation of the antigen in immunogenicity and in antigenic specificity, we have investigated the immune response to synthetic polypeptides of defined sequence and conformation (*26, 40, 58*). Thus, we can take the same tripeptide, L-tyrosyl-L-alanyl-L-glutamic acid, and either attach it to a branched polymer of alanine or polymerize it so as to get a polymer of high molecular weight of this tripeptide (Fig. 3). The branched polymer is an example of an immunogen with sequential determinants. On the other hand, the polymer of the tripeptide exists as an α-helix under physiologic conditions. We have used both polymers for immunization of rabbits and found that both are good immunogens. Thus, a structure which is completely α-helical can lead to an immune response. There is almost no cross-reaction between the two systems. Moreover, the tripeptide Tyr-Ala-Glu and related peptides are efficient inhibitors of the antibody-antigen reaction in the case of the branched polymer, but not at all in the case of the helical polymer. Thus, the specificity of the polymer of the above tripeptide seems to reside entirely in an area controlled by the α-helical structure of the polypeptide backbone, and is probably due to a particular juxtaposition of the amino acid side chains in the rigid conformation of the macromolecule.

When antibodies against the polymer $(Tyr\text{-}Ala\text{-}Glu)_n$ were reacted with a series of peptides of the formula ^{14}C-octanoyl-$(Tyr\text{-}Ala\text{-}Glu)_n$-Ala, in which n was 1, 2, 3, or 4, the tetrapeptide ($n = 1$) and the heptapeptide ($n = 2$) did not bind, whereas octanoylated $(Tyr\text{-}Ala\text{-}Glu)_3$-Ala bound well to the antibodies, and the octanoylated $(Tyr\text{-}Ala\text{-}Glu)_4$-Ala bound even significantly better. The last two peptides either already possess some helical conformation in solution, or they become transconformed into the helical form only after having reacted with the combining site of the antibody.

Gill and his colleagues (*59*) have explored the role of conformation in the structure of antigenic sites by investigating some synthetic polypeptides and their cross-linked derivatives. They concluded that there is no change in immunogenicity with change in conformation, but a marked change in the immunopotency of different antigenic determinants, as well as switch in specificity.

Efforts to obtain immunological cross-reactions between collagen and antibodies to poly-L-proline, and to polymers containing proline or hydroxyproline, were unsuccessful (*16*). We have studied (*26, 60*) a polymer of the tripeptide L-prolylglycyl-L-proline, which has the characteristic triple helix of collagen (*61*). Both guinea pigs and rabbits made antibodies against this polymer which cross-reacted with fish collagen, rat collagen, and even guinea pig collagen. This is the first instance that antibodies to a synthetic antigen reacted significantly with a natural protein, and clearly the reason for this reaction is due to their higher order structure. Thus, antigenic determinants of proteins are indeed controlled to a large extent by the secondary, tertiary, and quaternary structure of proteins.

Several other interesting cases of cross-reactions between antibodies to

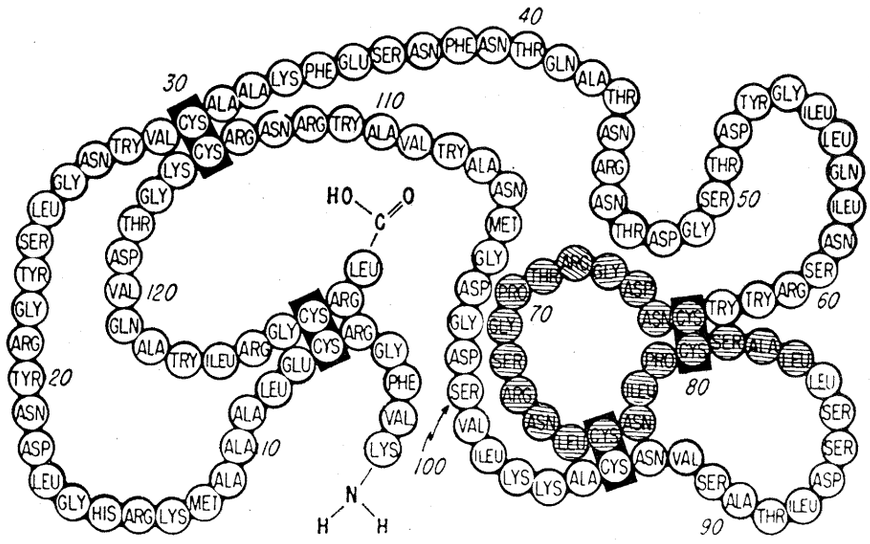

Fig. 4. Amino acid sequence of lysozyme from hen egg white. The region of the "loop" peptide is shaded (*65*).

synthetic polypeptides with natural antigens have been reported (*62*). Histone reacts with antibodies to poly-L-lysine. Copolymers containing glutamic acid, lysine, and alanine induced in rabbits the formation of hemolytic plaque-forming lymph node cells which cross-reacted with normal sheep erythrocytes. Antiserums against some synthetic copolymers containing D-alanine cross-react with a bacterial mucopeptide.

As the determinants of globular proteins are mostly conformation-dependent, their elucidation requires a detailed knowledge both of the amino acid sequence and of three-dimensional structure of the protein. Indeed, immunological studies of myoglobin (*63*) and of lysozyme (*64*) have pinpointed some immunopotent regions in these molecules. We have shown that antibodies reacting with a natural protein may be obtained upon immunization with a synthetic antigen conjugate (*65*).

A peptide containing the sequence 64 to 83 of lysozyme from hen egg white—and designated the "loop" peptide because it still contains one disulfide bridge (Fig. 4)—was attached to multichain poly-DL-alanine (multi-poly-DL-alanyl--poly-L-lysine). The resulting synthetic conjugate elicited in rabbits and goats the formation of antibodies with specificity directed against a unique region in native lysozyme. This was shown by the capacity of lysozyme to inhibit the homologous antigen-antibody reaction as well as by isolation of the antibodies capable of reacting with lysozyme on an immunoadsorbent prepared from lysozyme and bromoacetylcellulose (*65*). A small portion of the antibodies obtained upon immunization with lysozyme has a similar specificity, and these antibodies could be isolated with an immunoadsorbent prepared from bromoacetylcellulose and the "loop" peptide. In this case the capacity of the isolated antibodies to react with lysozyme is inhibited by the "loop" peptide, but not by the open-chain peptide derived from it after reduction and carboxymethylation. Thus, it seems that the antibodies to the "loop" are directed against a conformation-dependent determinant.

Recognition of Determinants

Many immunologists accept the hypothesis that ability to be split by hydrolytic enzymes is an important prerequisite for endowing a macromolecule with antigenicity. In view of the results pertinent to the role of steric conformation in the antigenic specificity of proteins and of synthetic polypeptides described earlier, the conclusion is unavoidable that no significant splitting by proteolytic enzymes may occur between the moment an immunogen is administered and the moment it is being recognized at the biosynthetic site. Such proteolysis would have to result in the destruction of the conformation of most protein determinants.

An additional argument for the intactness of the immunogenic molecule at the moment of recognition comes from a study on the role of the net electrical charge of the complete antigen in determining the chemical nature of antibodies to the *p*-azobenzenearsonate group (*66*). An inverse correlation between the net electrical charge on the antigen and that on the antibody it elicits in several animal species has been demonstrated in a series of studies (*67*). We have found now that most of the antibodies to *p*-azobenzenearsonate, obtained after immunization with the basic *p*-azobenzenearsonate-poly-L-lysyl-ribonuclease (with the positive charges being mainly on the outside of the molecule, and the *p*-azobenzenearsonate groups being more inside) were relatively acidic. In contrast, antibodies of the same specificity induced with the acidic *p*-azobenzenearsonate conjugate of rabbit serum albumin and of hexa-L-tyrosine, were of the more basic type. Thus, the antibodies formed reflected the overall net charge of the molecule, rather than the charge within the limited area around the *p*-azobenzenearsonate determinant in the immunogen. It may be concluded that the antigen control of the antibody type formed occurs at the level of the complete antigenic molecule.

It seems, therefore, that, if proteolytic destruction of the antigen plays a role in controlling the antibody formation, it would have to occur after the determinant has been "recognized" at the site of biosynthesis. The studies, mentioned above, on the role of the optical configuration in determining the antigenicity of synthetic polymers, are in agreement with this interpretation. Namely, the destruction of the immunogenic macromolecule, or at least of its antigenic determinants, may be necessary to prevent the overwhelming of the immune system with excess antigen which may obstruct antibody synthesis.

Other Specificities

Synthetic antigens may be prepared, leading to antibodies of almost any specificity desired (*2*). Not only have detailed studies of various immunological phenomena wtih the use of dinitrophenyl (*68, 69, 70*), *p*-azobenzenearsonate (*31, 71*), and penicilloyl (*72*) derivatives of synthetic polypeptides been made, but also synthetic immunogens have been prepared with determinants such as sugars (*73*), nucleosides (*74*), pyridoxal, folic acid, methotrexate (*75*), ferrocene (*76*), angiotensin, bradykinin (*77*), the aforementioned "loop" peptide of lysozyme (*65*), the naturally occurring glycolipid cytolipin H (*78*), and the phytoestrogen genistein (*79*).

Monosaccharides did not enhance the

immunogenicity of branched poly-DL-alanine (*73*), but all the nucleosides tested converted it into good immunogens (*74*), provoking in rabbits antibodies capable of reacting with RNA and single-stranded DNA. Antibodies with nucleotide specificity were obtained with photooxidation products of guanine-containing dinucleotides and a copolymer of lysine and glutamic acid (*80*). Antibodies to pyridoxal inhibited the enzymatic activity of aspartate aminotransferase (E.C. 2.6.1.1) (*75*). Antibodies against a copolymer of alanine, glutamic acid, and tyrosine to which lactosylsphingosine groups were attached reacted well with cytolipin H in which they recognized specifically both the sugar and the lipid moieties (*78*).

While most of the known immunogenic macromolecules are of a protein nature, it has been known long since that some polysaccharides may be immunogenic by themselves (*37*). Nucleic acids are usually not antigenic when administered alone, though the immunogenicity of transfer RNA has been described (*81*). Only in the last year there have been two reports on the limited antibody production to some nonmetabolizable synthetic polymers. These included polyvinylamine, polymethacrylic acid, polyvinylpyrrolidone (*82*), and mixed polycondensates (with relatively high molecular weight) of 4′4′-diaminodiphenylmethane-3,3′-dicarboxylic acid, and 4-nitro-2-aminobenzoic acid (*34*).

Genetic Control of Immune Response

The immune response to a specific antigen is a complex process that begins with the initial introduction of antigen into the animal and ends with the production of antibody. This process involves many steps, some of which may be under genetic control. All the steps involved in the immune response are not yet known, and we do not yet understand the genetic origin of antibody variability and specificity.

There is considerable evidence (summarized in *68* and *83*) that the immune response is under some form of genetic control, but in none of these earlier studies was a simple pattern of inheritance observed, probably because of the complexity of the immunogens used. Several studies, in which structurally simpler immunogens were used, have been recently reported. A single dominant autosomal gene determines whether or not guinea pigs can form antibodies to hapten-poly-L-lysine conjugates (*68*). The ability to respond does not depend in this case on the nature of the hapten, but on the nature of the polymeric backbone. This is the first system in which a strict unigenic control of an immune system was demonstrated and investigated in detail (*68, 84*). Genetic control of the capacity to produce antibodies against synthetic polypeptides has been shown also in mice (*83, 85–87*), rats (*88*), and rabbits (*89*).

Evidence for determinant-specific genetic control of antibody response in inbred strains of mice has been obtained with synthetic multichain polypeptide antigens in which short peptides containing glutamic acid and tyrosine, histidine, or phenylalanine were attached to the amino acid termini of a macromolecule containing poly-DL-alanine side chains linked, in turn, to a polylysine backbone (the tyrosine-containing copolymer is shown on the left side of Fig. 1). For example, C57 black mice are good producers of antibodies against the tyrosine-containing polymer and they respond poorly to the histidine-containing polymer; surprisingly, the situation is completely reversed in CBA mice (*83*). In short, the genetic factors can discriminate clearly between tyrosine, histidine, and phenylalanine in the determinant. The genetic differences are dominant, unigenic, quantitative, and determinant-specific.

The ability of mice to respond to the above antigens is a genetic trait which can be transferred with "responder" spleen cells, and which is closely associated with the major histocompatibility (H-2) locus in the IXth mouse linkage group (*86*). All strains of the same H-2 type exhibit the same pattern of immune response toward the above-mentioned three multichain antigens independently of the remainder of a given strain's genetic background.

When multichain polymers were built in which polyproline chains replaced the poly-DL-alanine side chains (*50*) and when their immunogenicity in inbred strains of mice was tested, the response was different (and not linked to the H-2 locus), even though the same short sequences of tyrosine or phenylalanine and glutamic acid were attached to the polypeptide side chains in both series, an indication that a portion of the alanine or proline sequences participates in the antigenic determinant (*87*).

Different inbred strains of mice may produce against the same protein similar amounts of antibodies, but this may be due to the complexity of the multideterminant antigen, so that the specificity of the antibodies formed may differ. For example, two different mouse strains (DBA/1 and SJL) immunized with the same antigen [poly (Phe,Glu)-poly Pro--poly Lys] responded equally well but with the production of antiserums of markedly different specificity (*87*). Antibodies formed in the DBA/1 strain cross-reacted well with poly (Phe,Glu)-poly DLAla--poly Lys and only weakly with poly (Tyr,Glu)-poly Pro--poly Lys. The opposite was true for the antibodies produced in the SJL strain. Thus, either the specificity of the antibodies produced or the recognition of antigenic determinants is under direct genetic control.

Role of Carrier

The immune response to one antigen may be inhibited as a result of the injection of a second antigen. This phenomenon, shown earlier for complex antigenic materials (*90*), and termed "antigenic competition," has been demonstrated recently also for defined antigenic determinants (*91, 92*) and for synthetic antigens (*93*). Whereas antibodies to poly-L-alanyl and poly-D-alanyl determinants are easeily elicited in rabbits and whereas the potency of the immune response toward either determinant is similar, immunization with poly-DL-alanyl, poly-DL-phenylalanyl, or poly-DL-tyrosyl protein conjugates led to the formation of antibodies directed mainly, or exclusively, toward D-amino acid sequences present in the determinant (*92*). We interpreted these results as competition between sequences composed of L and D residues.

Schechter studied the immunological competition between pairs of determinants attached separately to carrier molecules (*92*). The immune response toward the poly-DL-alanyl determinant was impaired by the poly-DL-phenylalanyl determinant only when identical or similar proteins served as the protein carriers of the singly substituted antigens, and not when, for example, a mixture of poly-DL-alanyl ribonuclease and poly-DL-phenylalanyl human serum albumin was used. In this case the nature of the carrier plays a crucial role in the competition between antigenic determinants.

The competition persists even in the tolerant state. Rabbits that were made tolerant in the neonatal state to poly-DL-phenylalanyl rabbit serum albumin would not produce antibodies with poly-DL-alanyl specificity when injected with a mixture of polyalanyl and polyphenylalanyl albumins, even though they were good producers of antibodies to the polyalanyl group when immunized with poly-DL-alanyl rabbit serum albumin alone. The nature of antigenic competition is still obscure. The specific competition described above seems a promising tool for the elucidation of this carrier-dependent phenomenon.

Polypeptidyl proteins and synthetic antigens were also used successfully in the study of immunological tolerance (*2, 9, 16, 94–96*). For example, tolerance to human albumin was terminated by the polytyrosyl, but not by the polyalanyl, derivative (*95*). We have also shown that neonatal injections of poly-DL-alanyl protein conjugates induced tolerance to the determinants of the native protein as well as to the polyalanyl group. A material must not necessarily be a good immunogen to induce effective tolerance, for we succeeded in inducing tolerance to the polyalanyl determinant with branched poly-DL-alanine.

There is no direct correlation between the specificity of antigen-antibody cross-reactions and the specificity of cross-tolerance (*96*). For example, an antiserum to the multichain poly (Tyr,Glu)-poly DLAla--poly Lys cross-reacts well with the linear copolymer of tyrosine and glutamic acid, and hardly at all with a uridylated poly DLAla--poly Lys. On the other hand, rabbits rendered tolerant to the first polymer were only partially tolerant to the linear copolymer and were completely unresponsive to the uridylated polymer (*9, 96*). It seems thus that the most immunopotent area of the molecule in terms of antibody formation is not necessarily the most important for the induction of tolerance, which may be mediated by areas in the "carrier" moiety.

The role of the immunological carrier, as defined earlier, in the control of the structure and biosynthesis of antibodies is especially clear from studies on the inverse correlation between the net charge of the antigen and that of the antibody it elicits (*9, 67*). Antibodies of the same specificity (dinitrophenyl, polyalanyl) which differ in their charge properties may be produced, depending on the chemical nature of the carrier in which the determinants were attached. The involvement of the carrier (defined somewhat differently by various authors) in the biosynthesis of antibodies has been mentioned in several other studies (*68, 97*) and has stimulated an additional theory of antibody formation (*98*).

Other Studies

Among relevant studies not discussed I would like to mention capacity of some tyrosine-containing linear and multichain copolymers to induce pure delayed hypersensitivity unaccompanied by antibody formation in guinea pigs (*99*), the effect of nutrition on the immune response toward synthetic antigens (*100*), the intracellular fate of highly radioactive antigens in the body (*101*), and the subcellular distribution of some synthetic antigens in the macrophages (*102*). In connection with studies on the involvement of an RNA-rich material from macrophages in the activation of lymphocytes to form antibody (*103*), it was shown that the RNA extracted from peritoneal macrophages exposed to a synthetic copolymer of glutamic acid, alanine, and tyrosine initiated an immune response in a strain of mice which responds very poorly to the antigen itself (*5, 104*).

Can a single cell produce antibodies of two different specificities? Studies with complex antigens, by a variety of methods, gave conflicting answers (*105*). No "double producers" were detected by fluorescent-antibody technique, when search was made for antibodies to the dinitrophenyl hapten and to the various determinants on the protein to which the hapten was bound (*106*). In a study looking for antibodies to two haptens, polyalanyl and *p*-azobenzenearsonate (linked to the same immunogen), by a modified Jerne hemolysin-plaque technique, no "double" plaques were found after counting 27,845 cells producing antibody (*105*), suggesting that there must be some mechanism which prevents a cell from synthesizing antibody of more than one specificity at a time. No conclusions can be drawn from the above experiments whether this is due to limited information in the genome or to some other regulatory mechanisms. From a study of the distribution of antibody-forming cells of different specificities in the lymph nodes and spleen of guinea pigs, Green (*106*) concluded that these cells may not arise by the antigen-stimulated proliferation of precommitted antibody-forming cells, but rather that antibody-forming cells arise by a transformation of uncommitted precursor cells.

Synthetic antigens may be of interest also as simple and well-defined immunogens which could lead to antibodies of restricted heterogeneity. Even isolated antibodies of a unique specificity are usually very heterogeneous, with the exception of myeloma immunoglobulins, for some of which antibody activity has recently been observed (*107*), and with the exception of antibodies with rhamnose specificity obtained after immunization of rabbits with streptococci (*108*). Less heterogeneous antibodies with dinitrophenyl specificity have also been obtained when the hapten was attached to a unique position in papain (*109*). Indeed, rabbit antibodies to a fully dinitrophenylated, defined sequence polymer, $[\text{DAla-LAla})_5\text{Lys}]_{10}$, bind radioactive dinitrophenyl-lysine with an energy which does not change later in the immune response, in contrast to antibodies obtained upon immunization with dinitrophenylated proteins (*70, 110*). This indicates that the antibody population showed some restriction in heterogeneity, even though the polymer exhibited no rigid conformation and probably possessed determinants of more than one dinitrophenyl specificity.

Summary and Conclusions

Our knowledge of many molecular parameters (such as composition, size, shape, accessibility, electrical charge, optical configuration, and steric conformation) controlling antigenicity, that is, immunogenicity and antigenic specificity, has increased considerably in recent years, and much of this advance is due to synthetic antigens. The relative simplicity of these molecules facilitates the interpretation of the results obtained with them, and sometimes permits the detection of effects, such as genetic variations in immune response, which are not easily observable with complex natural antigens. They are also helpful for our understanding of such other immunological phenomena as tolerance, antigenic competition, and delayed hypersensitivity. Studies of their intracellular fate and of their capacity to provoke primary and secondary immune response in vitro, combined with very sensitive

methods for antibody detection (*111*), should be instrumental for the elucidation of the mechanisms of antibody biosynthesis. Building of antigens with simple structure and unique conformation may lead to production of antibodies of a restricted heterogeneity.

The studies described here have led to the conclusion that an antigen is much more than just an antigenic determinant attached to an inert carrier, and that the carrier moiety of the molecule, while not contributing to specificity, plays an important role in defining the biosynthesis and structure of the antibodies formed. From a consideration of studies concerned with various aspects of antigenicity, it was also concluded that the recognition of the antigenic determinant occurs while the immunogen is still intact, even though ulterior destruction of the antigen—or at least of its determinants—may increase the efficiency of antibody synthesis.

Interest in synthetic antigens arose from their usefulness as model compounds, and ultimately it is in the natural antigens and their functioning that we are interested. Meanwhile, investigation of the immunological properties of the synthetic models seems rewarding in terms of the information on the basic principles and concepts of immunological mechanisms it has already yielded and of its potential applications in the future. Thus, the possible uses of such antigens for therapeutic purposes, while not yet considered, may become a reality in the foreseeable future.

When Arrhenius published *Immunochemistry* in 1907 (*112*), he stated in the preface that he did not believe that "the physiological side of the problem will find a satisfactory solution until the more simple chemical aspect is elucidated." Indeed, recent studies of antibody chemistry (*1*), as well as the studies on antigenicity described here, suggest that the progress of the "chemical aspect" has reached a stage where the elucidation of the biological mystery of antibody formation may soon be realized.

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113. Studies from our laboratory reported in this article have been supported by grants from NIH, the U.S. Air Force, and Minerva.