Outline Studies in Biology

Immunochemistry

M. W. Steward

OUTLINE STUDIES IN BIOLOGY

Editors : Professor T.W. Goodwin, F.R.S., University of Liverpool
Professor J.M. Ashworth, University of Essex

Editors' Foreword

The student of biological science in his final years as an undergraduate and his first years as a postgraduate is expected to gain some familiarity with current research at the frontiers of his discipline. New research work is published in a perplexing diversity of publications and is inevitably concerned with the minutiae of the subject. The sheer number of research journals and papers also causes confusion and difficulties of assimilation. Review articles usually presuppose a background knowledge of the field and are inevitably rather restricted in scope. There is thus the need for short but authoritative introductions to those areas of modern biological research which are either not dealt with in standard introductory textbooks or are not dealt with in sufficient detail to enable the student to go on from them to read scholarly reviews with profit. This series of books is designed to satisfy this need.

The authors have been asked to produce a brief outline of their subject assuming that their readers will have read and remembered much of a standard introductory textbook of biology. This outline then sets out to provide by building on this basis, the conceptual framework within which modern research work is progressing and aims to give the reader an indication of the problems, both conceptual and practical, which must be overcome if progress is to be maintained. We hope that students will go on to read the more detailed reviews and articles to which reference is made with a greater insight and understanding of how they fit into the overall scheme of modern research effort and may thus be helped to choose where to make their own contribution to this effort.

These books are guidebooks, not textbooks. Modern research pays scant regard for the academic divisions into which biological teaching and introductory textbooks must, to a certain extent, be divided. We have thus concentrated in this series on providing guides to those areas which fall between, or which involve, several different academic disciplines. It is here that the gap between the textbook and the research paper is widest and where the need for guidance is greatest. In so doing we hope to have extended or supplemented but not supplanted main texts and to have given students assistance in seeing how modern biological research is progressing while at the same time providing a foundation for self help in the achievement of successful examination results.

Immunochemistry

M.W. Steward
Kennedy Institute of Rheumatology, London

Chapman and Hall
London

First published in 1974
by Chapman and Hall Ltd
11 New Fetter Lane, London EC4P 4EE

Typeset by E.W.C. Wilkins Ltd., London and
printed in Great Britain by William Clowes & Sons Ltd.,
London, Colchester and Beccles

ISBN 0 412 12450 5

Distributed in the U.S.A.
by Halsted Press, a Division
of John Wiley & Sons, Inc. New York

Library of Congress Catalog Card Number 74–4104

Contents

1 Introduction

It has been known for a long time that contact with infectious organisms such as bacteria or viruses results in an individual becoming immune to subsequent re-infection with the same agent. Immunity to such infectious agents depends both upon the production of antibodies (γ globulins which react with the agent and assist in its elimination) *and* cell-mediated immune mechanisms. Whilst it is clear that these humoral and cellular aspects of immunity are interdependent it seems that modern research immunologists have segregated themselves into one or other of these fields. This dichotomy in immunology is not a new phenomenon. Indeed, it was not until the work of Wright (1903) showing that antibodies ('opsonins') actually aided the cell-mediated phagocytosis of bacteria described earlier by Metchnikoff (1883), that a compromise between the two hypotheses of immunity was achieved. However, whilst not aiming to condone or contribute to this segregation of research interests, the subject matter of this book is predominantly concerned with the humoral aspects of immunity; the cellular aspects will be dealt with in a further volume of this series.

The history of the study of antibodies goes back as far as 1890 when Von Behring first studied the neutralization of bacterial toxins by horse antitoxin antibodies. At about this time Paul Ehrlich, pioneer of immunochemistry became the first person to study quantitatively the precipitation reaction of toxin and antitoxin. It was his interest in immunological precipitation reactions which led the great physical chemist Arrhenius to be the first to use the word *immunochemistry*. In publishing in 1907 a series of lectures entitled 'Immunochemistry' he stated:

> 'I have given to these lectures the title 'Immunochemistry' and wish with this word to indicate that the chemical reactions of substances that are produced by the injection of foreign substances into the blood of animals, i.e. by immunisation, are under discussion in these pages. From this it follows also, that the substances with which these products react as proteins and ferments, are to be here considered with respect to their chemical properties.'

This definition is still applicable to modern immunochemistry and could be rephrased in modern terms as the study of the chemistry of antigens and antibodies and of the mechanism of their interaction.

Our concepts have of course, broadened from those of the early immunochemists who considered antibodies and antigens as homogeneous substances – even serum was considered as a single '*antigen*'. We now know that this is very far from the truth and that even simple purified antigens such as albumin have several antigenic determinants. The use of synthetic antigens of defined structure is helping to establish some of the molecular requirements for antigenicity. Antibodies are notoriously heterogeneous with regard to

structure and function and it is this fact which has hindered the detailed study of the chemical structure and synthesis of immunoglobulins. The discovery of myeloma proteins – homogeneous proteins representing a single immunoglobulin species in patients with myelomatosis – has greatly aided the study of antibody structure. Even so the question of whether myeloma proteins are truly representative of antibody still remains unanswered. One of the aspects of the immune response which has challenged immunochemists is the nature of the genetic control of such a system which enables antibodies to be produced with specificity towards a constantly changing antigenic environment. Whether the ability to exhibit such variation is a result of somatic mutation of a limited number of genes transmitted in the germ line, or whether the germ line carries all the necessary genes for antibody diversity remains to be elucidated.

In spite of the enormous advances made in the field of immunochemistry within the last few years, particularly in our knowledge of the structure, synthesis and function of antibodies and in preliminary studies of the location, structure and composition of the antibody binding site, much remains to be established.

It is against this general background that this book is written. Its aim is to give the reader a general outline of the considerable amount of immunochemical knowledge available and to convey something of the direction which current research is taking.

2 Antigens

The term 'antigen' is commonly used in two ways. Firstly to describe a substance which, when injected into an appropriate animal, elicits the production of circulating antibodies or changes in cellular reactivity such as delayed hypersensitivity, and, secondly, to describe a substance which has the property of reacting with antibody i.e. antigenic specifity. It is clear that these two characteristics are not the same. Some substances have the capacity to react with antibodies but do not themselves elicit antibody formation and similarly there are substances which provoke an immune response in some animals (responders) but not in others (non-responders). Thus in order to describe a substance according to its ability to induce an immune response, the immune responsiveness of the host must be taken into consideration. The term 'immunogen' has recently been used to describe this aspect of the antigenicity of a substance. Thus, in a responder, an antigenic substance is immunogenic whereas in a non-responder, it is non-immunogenic. The term antigenicity therefore defines two properties of a substance: (i) immunogenicity and (ii) antigenic specificity. Certain areas on the antigen molecule termed antigenic determinants are responsible for these properties of immunogenicity and antigenic specificity. These determinants have a three dimensional structure with which the antibody binding site reacts and an antigen may have several such determinants which are not necessarily identical.

Bacteria and blood cells were the most commonly studied antigens during the pioneering days of immunology. Subsequently bacterial toxins and other soluble plant and animal products were investigated and at the same time some idea of the chemical nature of these natural antigens was being obtained. These were generally classified as proteins, lipoproteins, glycoproteins or polysaccharides. The more recent application of sophisticated chemical and biochemical techniques for the isolation, purification and synthesis of antigenic substances has resulted in major advances in our understanding of the nature of antigens.

Antigens may be considered [1,2] as belonging to one of three classes: A. *Natural* antigens, B. *Artificial* antigens and C. *Synthetic* antigens (See Table 2.1)

Table 2.1 Classes of antigens

Class of Antigen	*Origin*	*Examples*
Natural	Plants, Bacteria, Animals.	Particulate: blood cells, bacteria, viruses. Soluble: toxins toxoids, proteins, carbohydrates, glycoproteins liproproteins.
Artificial	Chemically modified natural antigens.	Iodinated proteins, Protein-hapten conjugates. e.g. AZO & DNP – proteins.
Synthetic	Chemically synthethised molecules.	Polypeptides, Polyaminoacids, Multichain aminoacid copolymers.

These three broad categories of antigens will now be considered in outline, with examination of examples of antigens in each.

2.1 Natural antigens

2.1.1 Blood cells, viruses and bacteria

As mentioned above, the use of complex particulate natural antigens such as blood cells, bacteria and viruses has played an important role in the development of our understanding of the immune response. Indeed, such antigens as sheep red blood cells are still regularly used particularly in studies of antibody formation at the cellular level where the haemolytic plaque forming cell assay system of Jerne [3] facilitates the detection of antibody forming cells *in vitro*. The immunochemistry of red blood cell antigens is still the subject of a great deal of research interest both at the theoretical and applied level. With regard to the latter the study of the D red cell antigen (the antigen commonly involved in the haemolytic disease of newborn children) and of the kinetics of its reaction with the corresponding antibody has made a considerable contribution to the control of this disease by passive transfer of anti-D antibody to D negative (i.e. rhesus negative) mothers suspected of being sensitized with D positive cells [4].

The Tobacco Mosaic Virus (T.M.V.) was the first virus to be identified and crystallised and has been used as a multivalent antigen. The first electron microscopic demonstration of the antibody:antigen interaction employed this antigen. This virus is of particular interest to immunochemists since it consists of an RNA core and 2 130 identical polypeptide units (158 amino acids per unit of known sequence) as its protein coat.

The use of bacteria and viruses as antigens is still of considerable interest particularly where the chemical structure of the cell wall is known. For example the immunodominant group in the haemolytic streptococci groups A and C are known in detail (Section 2.1.3), and these antigens are being used in studies into the nature of the genetic control of antibody production.[1, 2, 3]

2.1.2 Proteins

Proteins (Table 2.2) were the first substances shown to be antigenic, and are still widely used as antigens.

Table 2.2 Commonly studied protein antigens

Protein	*Molecular weight (approx)*
TMV Subunit protein	17 000
Myoglobin	17 000
Flagellin	40 000 (& polymeric form)
Ovalbumin	44 000
Diptheria toxoid	65 000
Serum albumin	69 000
Transferrin	90 000
Globulins	170 000
Keyhole limpet haemocyanin	$2\text{-}7 \times 10^6$

Proteins are highly complex molecules which are readily obtained in a highly purified state from plants, animals and micro-organisms. However, very little is known about the precise nature of their multiple antigenic determinants. In spite of such limitations, studies employing protein antigens have made major contributions to our understanding of the immune response.

Attempts have been made to delineate some of the antigenic determinants of several proteins by limited proteolysis. Such a procedure yielded a peptide fragment from human serum albumin of molecular weight approx. 7 000 which contains one of the antigenic determinants of the parent molecule [5, 6]. Lack of detailed knowledge of the structure of proteins has hindered further study of this problem. However, myoglobin, a protein, whose amino acid sequence and conformation is known in detail, has been used for studies on the nature of its antigenic determinants. This protein which,

unfortunately is not a particularly good immunogen, has a minimum of four antigenic determinants. Immunochemical studies of the whole molecule and peptide fragments from it [7] have revealed that there are two types of antigenic determinants in the molecule (i) 'sequential': a determinant comprised of an amino acid sequence in a random coil form and (ii) 'conformational': a determinant whose nature depends upon steric conformation. These aspects will be discussed in more detail in Section 2.4.

Immunochemical studies of the protein antigen Flagellin – isolated from the flagella of *Salmonella* organisms have given some clues as to the nature and location of its antigenic determinants. Cleavage of the monomeric form (40 000 molecular weight) with cyanogen bromide (which breaks peptide bonds involving the amino acid methionine) yields four fragments, one of which contains all the antigenic determinants of the native molecule [8]. Further studies are needed to define more precisely the nature of these determinants. Similar studies have been carried out on the sub-unit protein of the Tobacco Mosaic Virus which has a molecular weight of approx. 17 000. The binding activity of peptide fragments of the unit protein with antibodies to the whole molecule have been studied in an attempt to localise the antigenically active site of the molecule.

In addition to the use of enzymically and chemically produced peptides for the study of the antigenic determinants on proteins, the technique of chemical modification has also been used. The effect of various chemical treatments on the antigenicity of the protein has been studied including denaturation, oxidation, reduction, digestion, deamination, esterification, acylation and halogenation [9]. Proteins treated in this way exhibit reduced reactivity with antibody to the native molecule, but interpretation of such results is a problem. The question of whether the effect is due to modification of a specific site or merely a conformational alteration as a result of the treatment is very difficult to answer.

It is clear that in spite of the wide use of proteins as antigens over many years, there is as yet very little information on the antigenic nature of these highly complex molecules and as such this field remains one of great challenge for immunochemists. Simple proteins and naturally occurring peptides have also been studies as antigens. These include insulin, glucagon, gastrin, calcitonin, bradykinin, angiotensin, vasopressin, adrenocorticotrophic hormone and growth hormone. In general, they are poor immunogens and require that their immunogenicity be enhanced in order to induce antibody formation either by immunization with adjuvants or by chemical coupling to natural or synthetic carrier molecules. Such poor immunogenicity has been ascribed to the low molecular weight of the peptides. The measurement of several of these substances in human serum by radioimmunoassay has useful clinical application. Their poor immunogenicity poses quite a problem since such assays require a potent (high affinity) antibody in order to be sufficiently sensitive.

2.1.3 Carbohydrates

Most types of bacteria have serologically active carbohydrates on or in their cell walls. Such carbohydrates react with antibody raised to the bacteria but are not themselves immunogenic i.e. they behave as haptens (See Section 2, 2). In other cases isolated carbohydrates such as the group A and group C carbohydrates of meningococci and the pneumococcal polysaecarides are immunogenic in man. Other carbohydrates such as dextrans, levans and teichoic acids have also been shown to be immunogenic in man. The molecular weight of the carbohydrate appears to be particularly important in the immunogenicity of these

substances with repeating antigenic determinants. Dextrans – polymers of D-glucose, have been widely studied in this regard and it has been shown that dextrans of molecular weight less than 50 000 are far less immunogenic in man than those of molecular weight 90 000 or above [10]. The molecular weight of type III pneumococcal polysaccharide has to exceed 18 000 to induce an immune response in mice [11]. The group specific carbohydrates A and C from streptococcal cell walls have been extensively studied as antigens. The immunization of rabbits with vaccine prepared from heat-killed, pepsin digested Group A or Group C organisms elicits the formation of antibody to the group specific carbohydrate. The group A carbohydrate consists of repeating N-acetyl glucosamine-rhamnose units (17 moles of N-acetyl glucosamine and 38 modes rhamnose) and has a molecular weight of approximately 10 000. The N-acetylglucosamine is the immunodominant group. Group C carbohydrate has a similar structure consisting of N-acetylgalactosamine-rhamnose units with N-acetylgalactosamine as the immunodominant moiety. The exquisite specificity of the antibodies formed is illustrated by the fact that N-acetyl glucosamine inhibits the precipitation of A carbohydrate by antibody to A vaccine, whereas N-acetylgalactosamine does not. The converse is true with the C carbohydrate [12]. The group specific streptococcal and pneumococcal carbohydrates are particularly interesting to immunochemists because rabbit antibodies to such carbohydrates are, unlike normal antibodies, frequently of restricted molecular heterogeneity [11]. The study of such antibodies is making a major contribution to our knowledge of the genetic control of immunoglobulin structure and synthesis.

2.1.4 Lipids

The immunogenicity of a purified lipid has never been convincingly demonstrated and it appears that in order to obtain antibody to a lipid, it has to be complexed with larger macromolecular structures such as proteins, synthetic polypeptides or red blood cells.

2.1.5 Nucleic acids

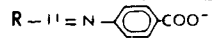
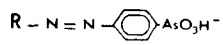
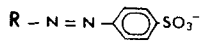
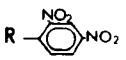
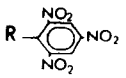
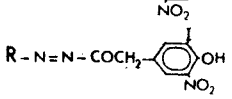
Within the last two decades, nucleic acids have been of great interest to biochemists, and the finding of antibodies to nucleic acids in the serum of patients with the disease *systemic lupus erythematosus* (SLE) aroused the interest of immunochemists in these complex molecules. SLE is a disease with numerous immunological abnormalities including the presence of antinuclear and antinucleic acid antibodies in the serum. Immune complexes of anti DNA antibody and DNA are frequently deposited in the glomeruli of the kidneys. Antibodies to nucleic acid antigens have been sucessfully obtained experimentally only after conjugation to carriers such as methylated bovine serum albumin, proteins or synthetic polyamino-acids. In spite of considerable interest in the immunochemistry of these materials the precise nature of their immunogenic properties remains to be defined.

2.2 Artificial antigens

It will be clear from what has been said above that in order to gain knowledge of the chemical basis for immunogenicity, the problem of the enormous complexity of natural antigens has to be overcome. One approach which has been used by immunochemists to answer this problem is that of chemical modification of natural antigens to produce artificial antigens. Thus, the substitution of small determinant groups of known chemical structure on to protein antigens has provided much of our information on the nature of the specificity of immunological reactions.

It has been known since the early part of this century, that proteins lose their species specificity when heavily iodinated. Antibodies

Table 2.3 Examples of common haptens

Group	*Structure*	*Mode of coupling to carrier*
azobenzoate		azo bonds with aromatic rings of tryosine, histidine, and ϵ-NH_2 of lysine
azobenzenearsonate		
azobenzensulphonate		
dinitrophenol		nucleophilic substitution using halogen derivative, i.e. 2, 4-dinitrofluoro-benzene
trinitrophenol		NH_2 groups of lysine
4-hydroxy-3-iodo-5-nitrophenacetyl		azide reacts with NH_2 groups of lysine
R = protein	R = protein	

to such iodinated proteins are directed mainly to the iodinated tyrosine residues common to such iodinated proteins. However, it was not until the classical immunochemical experiments of Karl Landsteiner [13] using well defined small molecules coupled to proteins, that information regarding the specificity of immunological reactions began to accumulate. Landsteiner first introduced the term 'hapten' to describe 'specific protein free substances which, although reactive *in vitro*, induced no, or only slight antibody response. For serologically active substances of this sort, in contradistinction to the protein antigens, which possess both properties, the term *'hapten'* has been proposed'.

Although the term 'hapten' is frequently applied to low molecular weight aromatic substances (Table 2.3), the above definition indicates that the term can be applied to any substance which does not elicit antibody formation by itself (i.e. is not immunogenic) but is capable of reacting with antibodies synthesised against a complete immunogenic molecule. Substances which are as varied as aromatic compounds, certain drugs (Penicillin), oligosaccharides, nucleic acids and nucleotides, peptides and lipids can all be haptens.

Although the role of conformation of configuration in immunogenicity will be discussed in more detail in section 2.4, the pioneering work of Landsteiner on the serological specificity of different azo-proteins will be mentioned briefly here. He demonstrated that when the three isomers of aminobenzene sulphonic acid (ortho, meta and para) were diazotized and coupled to protein (horse serum protein) and injected into rabbits, the antibodies so produced were able to differentiate between the three isomers. Thus antibodies to the horse protein containing the meta azobenzene sulphonate group gave a good precipitate with a

different carrier protein bearing the meta isomer, but only poor precipitates with the two other isomers. In similar experiments, Landsteiner and Van der Scheer [14] were able to demonstrate by inhibition of precipitation, that the antibody to the sulphonic acid hapten could distinguish between the sulphonic acid hapten and an azobenzene hapten in which the sulphonate group was replaced by an arsonate or carboxylate group. These experiments further demonstrate the exquisite specificity of the antibody-antigen interaction.

The use of azocoupling to produce conjugates is not only applicable to aromatic amines but can be used to couple carbohydrate derivatives to proteins. Indeed, Landsteiner was able to demonstrate the serological specificity of D- and L-tartaric acids by synthesising the two isomeric diazo-tartranilic acids and coupling these to carrier protein. Antibodies to these conjugates could distinguish between these and the D- and L-isomers. Such work is an excellent example of the contribution that studies on artificial antigens have made to our knowledge of the specificity of the immunological response.

Before going on to discuss the use of completely synthetic antigens, the results of studies in which chemical groups were attached to the poor immunogen gelatin in order to investigate their effect on its immunogenicity will be briefly considered. The results of several studies are summarized in Table 2.4 and detailed references can be found in [15]. It is clear that the addition of a wide range of chemical groups can generally enhance the immunogenicity of gelatin. When the aromatic amino acid tyrosine is incorporated at a level of 2% into gelatin, the immunogenicity of gelatin itself is greatly enhanced. However, at levels of 10% tyrosine, the gelatin is again highly immunogenic but the specificity of the antibodies produced is directed towards tyrosyl peptides and not to gelatin [1]. Such studies, although providing some indication of the nature of immunogenicity, have obvious limitations. The introduction of techniques to produce completely synthetic antigens has led to a rapid advance in our understanding of the molecular basis of immunogenicity.

Table 2.4 The effect of coupling haptens and peptides on the immunogenicity of gelatin

Group coupled	*Effect of immunogenicity*
Sugar residues	
e.g. arabinose	–
cellulose-glycol	–
Sugar residues + tyrosine	†
Aromatic compounds:-	
e.g. diazonium compounds	†
benzoic acid	–
phenylisocyanate	–
Amino Acids:-	
D-tyrosine, *L*-tyrosine	†
phenylalanine, methionine,	†
tryptophan lysine, glutamine, alanine, serine	–
Polyamino Acids:-	
polytyrosine, polyphenylalanine, polytryptophan, polyglycine	†
polyalanine, polyglutamic acid, polylysine	–
Peptides:-	
leucine – glutamic acid, lysine-glutamic acid, tyrosine – glutamic acid	†

† = increased immunogenicity
– = no effect on immunogenicity

2.3 Synthetic antigens

From the preceding discussion it will be clear that studies into the precise chemical nature of what confers the immunogenic and antigenic properties to natural antigens such as proteins has been hampered by their extreme complexity. Valuable information on the specificity of the immune response has been

obtained using artificial antigens but it was the availability of synthetic linear and branched polymers and copolymers of amino acids during the last 10–15 years, which has contributed greatly to our understanding of the molecular basis of immunogenicity. By studying the effects of precise changes in the chemical structure of these antigens, it has been possible to delineate many of the molecular characteristics which influence immunogenicity. Many such synthetic antigens have been tested in several species including mice, rabbits, guinea pigs, rats and humans and review articles should be consulted for detailed information. A summary of the types of synthetic antigens used in immunochemical studies is shown in Table 2.5.

Table 2.5 Synthetic polypeptide antigens

Type	*Example*
Homopolymer	poly L-pro
Linear polypeptide	poly glu^{56} lys^{38} tyr^{6}
Random copolymer	(pro^{66} gly^{34}) n
Ordered sequence or periodic polymer (α helix)	(tyr-ala-glu) n
Multichain (branched) copolymer	poly (tyr-glu)-poly-DL-ala-poly-lys

An example of the structure of an immunogenic multichain (branched) copolymer poly (try-glu)-poly-DL-alanine-poly-*L*-lysine used by Sela [1] is shown in Figure 2.1(a). Here, tyrosine and glutamic acid residues are attached to the external part of the poly-DL-alanine-poly-L-lysine backbone (which by itself is not immunogenic). If the same amino acids are attached to the poly-L-lysine backbone directly and the poly-DL-alanine peptide then added (Fig. 2.1(b)) the resulting polypeptide is non-immunogenic. This illustrates the need for the immunologically important groups to be accessible in order to elicit antibody formation. Work from the same author's laboratory has also shown that the same tripeptide (L-tyr-ala-L-glu) can be synthetically made either into a branched copolymer (an immunogen with sequential determinants) by attaching it to the side chains of poly-DL-ala-poly-L-lysine (Fig. 2.2(a)) or into an ordered sequence (or periodic polymer) which exists as an α-helix giving an immunogen with conformational determinants. (Fig. 2.2(b)). Antibodies to the sequential determinants do not cross react with the conformational determinants and vice versa, showing the importance of conformation in immunogenicity and antigenic specificity.

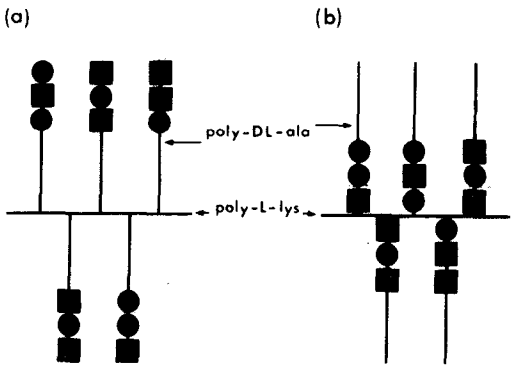


Fig. 2.1 Multichain copolymers of poly (tyr, glu)-poly-DL-ala-poly-L-lys. (a) immunogenic form (b) non-immunogenic form. (After [1]).

These examples serve to illustrate the type of studies which can be carried out with synthetic antigens. The kind of information obtained from such investigations on the molecular basis of immunogenicity and antigenic specificity will be summarized in the following section.

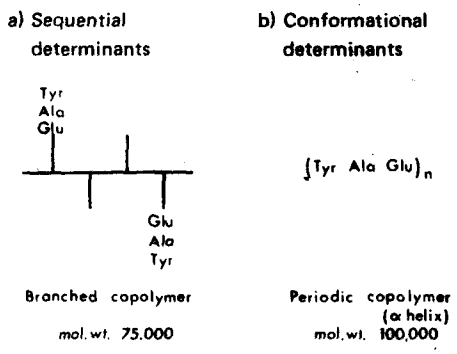


Fig. 2.2 Synthetic sequential and conformational antigenic determinants. (After [1]).

2.4 Molecular basis for immunogenicity and antigenic specificity

Sela has said [1]

> '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 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.'

In this section our present understanding of the role of the above parameters in controlling antigenicity will be briefly summarized.

2.4.1 Size

At one time it was thought that molecular weights below 5–10,000 were not immunogenic. This is probably true for protein antigens but other molecules of molecular weight as low as 450 have been shown to be immunogenic (Table 2.6) either as evidenced by tests for antibody formation or alteration in cellular reactivity.

Table 2.6 Immunogens of low molecular weight

Immunogen	*Molecular Weight*	*Species immunized*
poly-Glu^{50}-Ala^{40}-Tyr^{10}	4 000	rabbit
tri-dinitrophenyl bacitracin	1 928	guinea pig
Angiotensin	1 031	guinea pig
p-azobenzenearsonate-tri-L-tyrosine	750	rabbit, guinea pig
p-azobenzenarsonate-N-acetyl-L-tyrosine	451	guinea pig

2.4.2 Composition

Homopolymers of α-amino acids are not generally immunogenic but polymers of two amino acids are immunogenic in rabbits and guinea pigs. Excellent immunogens have been obtained with polymers consisting of three or four amino acids. The coupling of tyrosine and phenylalanine to gelatin greatly increases its immunogenicity (Section 2.2). Similar effects are obtained using cyclohexylalanine (in which the aromatic rings of phenylalanine are replaced by cyclohexane rings) indicating that it is the ring structure rather than the aromaticity which makes tyrosine and phenylalanine immunogenically important. The presence of aromatic amino acids such as tyrosine in synthetic polypeptides, although generally increasing the amount of antibody produced, is not critical for their immunogenicity.

2.4.3 Steric conformation

It has been recognised for a long time that the spatial arrangement of proteins is important in antigenic specificity since denatured proteins react poorly, if at all, with antibodies directed to the native molecules. Work with synthetic