Antigens and Antigenicity

MICHAEL SELA*

Department of Chemical Immunology,
The Weizmann Institute of Science, Rehovoth, Israel

The topic of this lecture is antigenicity. Now, whether we inject bacteria or viruses to obtain immunity against them; whether we unintentionally are making antibodies to a protein present in strawberries, shrimps or almonds; whether our organs are rejected after transplantation; or whether we start making antibodies against our own body constituents, in all these cases there is a common reaction mechanism, even though the effects are so widely different in terms of their meaning for health and disease. We always have an antigen leading to an immunological response, be it antibody formation, or be it cellular response devoid of the presence of humoral antibody in the bloodstream, as is the case of delayed hypersensitivity.

While initially the word "antigen" meant a substance capable of provoking an immune response in man or in an experimental animal, it became used more and more also in a different context, namely, as a material which can react immunospecifically with antibodies. To give you one such example: immunization with *Bacillus anthrax* leads to antibodies which react well with a polymer isolated from the cell wall of this bacterium, the poly-γ-D-glutamic acid [1]. If one immunizes with this poly-γ-D-glutamic acid, no antibodies are formed at all. Thus, this material is only a cross-reacting antigen.

I would like, therefore, for the sake of clarity, to distinguish between two separate notions [2]. By immunogenicity I understand the capacity of a material to provoke an immune response and this notion is not related at all to specificity. On the other hand, antigenic specificity is a separate property. If I may come back to the example I have just mentioned, poly-γ-D-glutamic acid is not an immunogen, whereas *Bacillus anthrax* is immunogenic and leads to the formation of antibodies specific to poly-γ-D-glutamic acid.

One talks usually of bacteria, viruses, or even animal organs and tissues, as antigens but they are really multi-molecular poly-determinant complex mixtures of antigens. Even one protein molecule is still a complex poly-determinant antigen.

Until recently, all the antigens known were of a natural origin, be it microbial, plant or animal. The efforts to understand better the specificity of immunological reaction led the immunochemists to modify chemically such natural antigens. I would like to call these modified materials — *artificial antigens*. This approach has been mainly due to the pioneering work of KARL LANDSTEINER who succeeded to demonstrate beautifully the great specificity of serological reactions by using well defined chemically small molecules which he attached to proteins, and thus changed immunological specificity of these immunogens [3]. It is particularly pertinent to mention here today the name of KARL LANDSTEINER as this year is the 100th year of his birth. He not only discovered the blood groups but he also led the basis for modern immunochemistry.

We may draw a parallel with enzymes, where a certain area may be assumed to possess catalytic activity, while a different area of the molecule — even though sometimes it may overlap or be identical with the first one — is responsible for the enzymic specificity. In the case of antigens it seems desirable to discuss, separately, the moiety that must be present in the molecule to render it immunogenic, and a different moiety responsible for the serological specificity. Besides the natural and the artificial antigens — which I have already defined — I would like to mention the synthetic antigens. While the artificial antigens are immunogenic to begin with and we can learn from them mainly about antigenic specificity, using synthetic molecules may help us to get a deeper understanding both about the reasons for immunogenicity and the reasons for specificity. Thus, we may ask ourselves the question: What is really an antigen?

The Molecular Basis

Being interested in defining the minimal conditions necessary to give a molecule immunogenic properties, we chose in our initial work gelatin as an example of a protein molecule which is a very poor immunogen. As a matter of fact, when we started working with it over a dozen years ago [4] it was still believed to be essentially non-immunogenic. And it was thought that it was not immunogenic because it does not contain tyrosine. We know today that gelatin contains some tyrosine, though very little, and that it is an immunogen, even though a very poor one. We asked ourselves, therefore, whether we can enhance the immunogenicity of gelatin by attaching to it tyrosine peptides. This was achieved by reacting gelatin with the N-carboxy-L-tyrosine anhydride.

The polytyrosyl gelatin obtained was found to be a good immunogen in guinea pigs and in rabbits. Thus, attachment of tyrosine has enhanced dramatically the immunogenicity of gelatin [5]. We have checked most of the natural amino acids for their capacity to increase the immunogenicity of gelatin, and found that the attachment of phenylalanine and of tryptophan also converted gelatin into potent immunogens. This is not necessarily due to the aromatic character of these amino acids, as the attachment of cyclohexylalanine, an amino acid obtained by hydro-

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genation of the aromatic ring of phenylalanine, also enhanced immunogenicity [6].

The amount of antibodies provoked by gelatins enriched with tyrosine to a different extent is shown in Fig. 1. As little as 2% of tyrosine is enough to convert gelatin into a good antigen. Five percent tyrosine leads to a better response, which is not significantly different from the response to gelatin enriched with 10% tyrosine. Now, what about the specificity of the antibodies formed? We found out that the antibodies against the material with 2% tyrosine added were directed mainly against gelatin, and they precipitated well with gelatin. On the other hand, antibodies to the material with 10% tyrosine added did not react at all with gelatin, whereas they reacted with other polytyrosyl proteins [7]. It is obvious that no antibodies were made in this case against gelatin determinants. Does it still mean that there is something essential about the presence of gelatin in this immunogenic molecule? Or can we replace it with a well-defined chemically synthetic molecule? To answer these questions we have decided to replace gelatin with a synthetic branched polypeptide of polyalanine [8, 9].

For its synthesis we used poly-L-lysine as the multifunctional initiator and we grew on every ε-amino group a polymeric side chain of DL-alanine (Fig. 2). I must mention here, parenthetically, that polymers of L-alanine, or polymers of D-alanine, exist in an α-helical form and are utterly insoluble in water. In contrast, polymers of DL-alanine, which are really copolymers of L-alanine and of D-alanine, dissolve very well in water. The branched poly-DL-alanine is a very poor immunogen, if at all. We have used it as a multifunctional initiator for the polymerization of tyrosine, making use of the terminal α-amino groups, and we added also the monomer of glutamic acid for solubility reasons (Fig. 2, left). This polymer is a good immunogen in rabbits, guinea pigs and mice and leads to the formation of antibodies of a very narrow and well-defined specificity [9—12]. Thus we had here the first instance of a completely synthetic macromolecule which is a good and specific antigen. Since then tens of analogues of these synthetic antigens, as well as many other linear and branched polymers of amino acids, have been prepared and tested in rabbits, guinea pigs, mice, rats, goats, monkeys and humans [2, 13, 14]. Several groups of investigators, including STAHMANN, MAURER, GILL and DOTY, as well as FUCHS and ARNON in our laboratory, have been engaged in this work. The aim is to track down, by controlled changes in the chemical structure, those features that are connected with antigenic activity.

Let us take stock of what has been learned so far from the study of synthetic antigens. For example, we could build a molecule which will have the same size, shape and composition as the one mentioned above (Fig. 2, left). But now we have put the peptides of tyrosine and glutamic acid on the inside, and we have covered them with a thick fur of polyalanine (Fig. 2, right). The first material was a very good immunogen, whereas this one is not immunogenic at all. I should mention that the material before the attachment of polyalanine to the outside was already a good immunogen. We see thus that we can convert by chemical modification a non-immunogenic molecule into an immunogenic one and, vice versa, an immunogenic molecule into a non-immunogenic one. We can conclude from this experiment also about the importance of the accessibility of the antigenically important part of the molecule to the biosynthetic site of the antibody.

Concerning the composition, the story is not so simple. For example, it turns out that one cannot classify the amino acids simply into those that can make antigens and those that cannot. It depends on the circumstances. The attachment of glutamic acid alone, or lysine alone, will not convert branched polyalanine

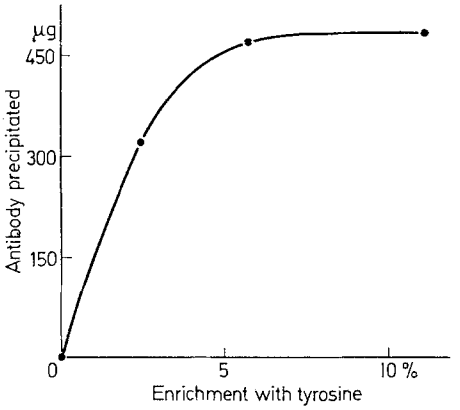

Fig. 1. Maximal amounts of antibodies precipitated in 1 ml of serum, from antisera against several poly-L-tyrosyl gelatins and against gelatin, as a function of the extent of the enrichment of gelatin with tyrosine residues. From ARNON and SELA [7]

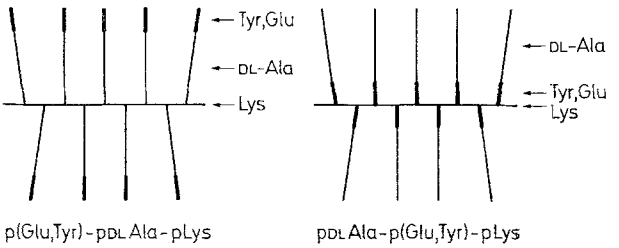

Fig. 2. Schematic representation of a multichain copolymer in which L-tyrosine and L-glutamic acid residues are attached to multi-poly-DL-alanyl—poly-L-lysine (left), and of one in which tyrosine and glutamic acid are attached directly to the lysine backbone and then elongated with alanine peptides (right)

into a good antigen, but attachment of a mixture of the two will [15]. Similarly, copolymers of lysine and glutamic acid are antigenic, but polylysine and polyglutamic acid separately are not [16, 17]. If a polymer of two amino acids is not antigenic, maybe one of three will be. You see how easy it would be to become a kind of immunological computer, and I do not think this is a worthwhile approach, especially in view of the fact that in those antigens which interest us most, like the proteins, the immunogenicity and the specificity are controlled primarily by their higher order structure, rather than by their amino acid components.

The interesting question is what is the smallest molecule that can have antigenic properties. The lower limit for immunogenicity has been considered in the range of 5 to 10 thousand. We have worked with a linear polymer with a molecular weight of

4000 which was still a very good immunogen [9]. But more recently several much smaller molecules have been shown to be immunogenic (Table). Thus, for example, a hexapeptide — or even a tripeptide — of tyrosine, to which one diazonium salt derived from arsanilic acid has been attached, are capable of provoking a good immune response in guinea pigs and in rabbits.

The role of electrical charges in binding the antigen to the antibody and in determining antibody specificity is a question which had been studied with natural and artificial antigens. The natural forms usually do contain some charged groups, but with the advent of synthetic antigens it was possible to check whether the charges were essential for antigenicity. They were found not to be, since a certain synthetic polypeptide containing tyrosine proved to be devoid of charges in neutral aqueous solutions but was nevertheless a good antibody producer in rabbits [24].

Table. *Some low molecular weight immunogens*

Designation	Molec. weight	Species	Lit.
Angiotensin	1031	Guinea pig	[18]
Tri-dinitrophenyl-bacitracin	1928	Guinea pig	[19]
α-Dinitrophenyl-hepta-L-lysine	1080	Guinea pig	[20]
"Arsanil"-hexa-L-tyrosine	1200	Rabbit Guinea pig	[22] [21]
"Arsanil"-tri-L-tyrosine	750	Rabbit Guinea pig	[22] [21]
"Arsanil"-N-acetyl-L-tyrosine amide	450	Guinea pig	[21]
"Arsanil"-N-acetyl-L-tyrosine	451	Guinea pig	[23]
"Arsanil"-N-acetyl-D-tyrosine	451	Guinea pig	[23]

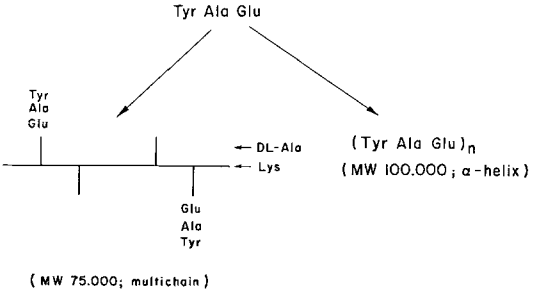

Fig. 3. Schematic representation of a synthetic branched polymer in which peptides of sequence TyrAlaGlu are attached to the amino termini of polymeric side-chains in multi-poly-DL-alanyl—poly-L-lysine (left), and of a periodic polymer of the tripeptide TyrAlaGlu (right)

The synthetic antigens investigated have well-defined specificities, as shown by cross-precipitation and inhibition experiments. Thus, antibodies formed against peptides of tyrosine and glutamic acid attached to branched polyalanine will react with the same peptides attached to gelatin as well as with a linear copolymer of tyrosine and glutamic acid, but will react hardly at all with the parent branched polyalanine [8, 10]. A detailed analysis of the specificity of some anti-polypeptide antibodies has led us to conclude that the combining site on the antibody molecule is such as to accommodate a peptide of four amino acids [25, 26].

Conformation and Specificity

A subject with which we were concerned more recently is the role of conformation in immunogenicity and in antigenic specificity [27]. It has long been known that the spatial folding up of proteins plays an important role in determining their antigenic specificity. Thus, antibodies to native proteins react poorly — if at all — with the same proteins after denaturation, because the unique spatial conformation of the native material has been destroyed. Undoubtedly, this phenomenon is due to changes within the conformation of the protein molecule. "Conformation" designates here a particular arrangement of atomic positions of a molecule, which can be achieved without the reorganization of chemical bonds. The unique conformation of a native protein is the result of its primary, secondary, tertiary, and — when applicable — quaternary structure. The primary structure is the chemical sequence of amino acids within a polypeptide chain; the secondary structure results from interactions between the polypeptide backbones, usually involving hydrogen bonds (e.g., α-helix, β-structure); the tertiary structure is concerned with intrachain and interchain interactions between amino acid side chains, and includes disulfide bridges; finally, the quaternary structure results from interactions between protein subunits.

An antigen can provoke antibodies against many different determinants present in its molecule, and some of them may be overlapping. Thus, antisera 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.

A "sequential" determinant is 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.

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. Thus, we can take the same tripeptide and either attach it to a branched polymer of alanine or polymerise it so as to get a high molecular weight polymer 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, an α-helical structure suffi-

ces to lead to an immune response. There is almost no cross-reaction between the two systems. Moreover, the tripeptide TyrAlaGlu 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 tripeptide TyrAlaGlu 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 [27].

We have carried out a similar study of the polymer of the tripeptide prolyl-glycyl-proline, a polymer which has been shown previously to have the characteristic triple helix of collagen [27]. 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 react with a natural protein, and clearly the reason for this reaction is due to their higher order structure. We see thus, that antigenic determinants of proteins are indeed controlled to a large extent by the secondary, tertiary and quaternary structure of proteins.

In a recent study with RUTH ARNON we have shown that antibodies reacting with a natural protein may be obtained upon immunization with a synthetic antigen conjugate [28]. We have cut out with pepsin the part of the lysozyme molecule (Fig. 4) which is the amino acid sequence 64—83, and we have attached it to the branched polyalanine. Immunization of rabbits with this conjugate led to the formation of antibodies which reacted with lysozyme. As these antibodies react with only one region within the lysozyme molecule, they obviously could not give any visible precipitates, but we could prove the reaction by showing the capacity of these antibodies to bind radioactive lysozyme. More conclusively, we have bound lysozyme chemically to cellulose, we have reacted this lysozyme-cellulose immunoadsorbent with antisera to the synthetic conjugate and thus have adsorbed most of the antibodies, eluted them with acetic acid, and obtained immunospecific antibodies. On the other hand, one can also inject rabbits with lysozyme and pick up from the anti-lysozyme antisera only those antibodies which react with the same region of the lysozyme. This was done by binding the peptide 64—83 to cellulose, and fishing out by means of these immunoadsorbents the antibodies specific to the peptide. Thus we have two preparations of antibodies, both reacting with lysozyme, both reacting with one unique region of lysozyme, but one of them was prepared by immunization with lysozyme and the other one by immunization with a synthetic conjugate. It should be of interest to compare the properties of these two antibodies.

I shall not discuss the optical configuration of the amino acids, except to say that peptides of D-amino acids are as good antigenic determinants as peptides of L-amino acids, but, as far as immunogenicity is concerned, polymers of D-amino acids are very poor immunogens and induce easily immunological paralysis [30, 31].

If one considers both the results I have described of the steric conformation, and the recent studies on the role of optical configuration, it is unavoidable to draw the conclusion that no significant splitting by the proteolytic enzymes may occur between the moment an immunogen is administered and the moment it is being recognized at the biosynthetic site. Otherwise, the conformation of the protein determinant would be destroyed by proteolysis. On the other hand, it seems that proteolysis may be important, and its role is to destroy the antigen *after* the determinant has been recognized at the site of biosynthesis.

Other Specificities

The synthetic antigens described so far were composed solely of amino acids. But the synthetic approach is not limited to antigens with peptide specificity. It is possible to attach small haptens such as the dinitrophenyl group, or the diazonium salt

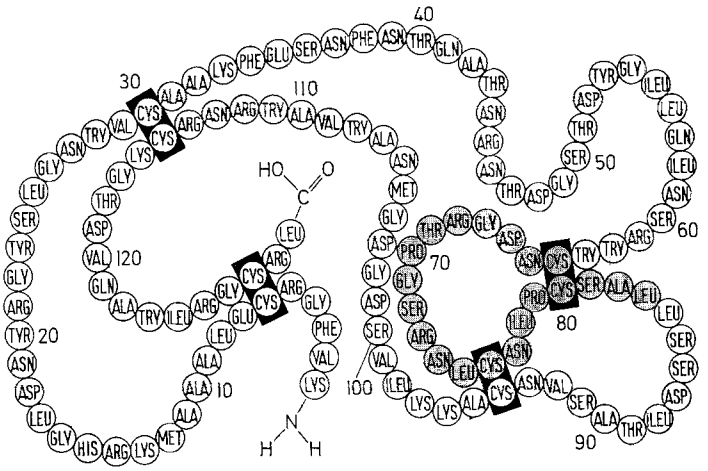

Fig. 4. Amino acid sequence of hen egg-white lysozyme [29]. The region of the "loop"-peptide is shaded. From ARNON and SELA [28]

derived from arsanilic acid, or penicillin derivatives, to synthetic polypeptides, getting e.g., penicilloyl polylysine, which provokes the formation of anti-penicillin antibodies [2].

We have shown, in collaboration with Prof. WESTPHAL and Dr. RÜDE of the Max-Planck-Institut für Immunbiologie in Freiburg, that after glucose is attached to a synthetic antigen the antibodies elicited have mainly glucose specificity [32]. Glucose was attached as glucosyl serine either to a synthetic antigen which already contained tyrosine, or to the non-antigenic branched polyalanine. The attachment of glucose to the branched polyalanine did not convert it into a good antigen, whereas antibodies to the conjugate of glucose with the synthetic immunogen were almost entirely specific to glucose. It thus seems that in this case a sugar can only serve as a specificity determinant and not as an enhancer of antigenicity.

In contrast to this observation, the attachment of nucleosides to the branched *polyalanine*, converts them into good immunogens [33]. Nucleosides are converted by catalytic hydrogenation with molecular oxygen into nucleoside 5'-carboxylic acids.

These may be attached by means of carbodiimide reagents to the amino termini of the polyalanine side chains (see, e.g., Fig. 5). All these derivatives were good immunogens and led to very specific antibodies. Thus, for example, anti-uridine antibodies reacted with uridine and to some extent with deoxyuridine and thymidine, but they did not react with pseudouridine or dehydrouridine. Anti-uridine antibodies did not react with uracil or ribose, or a mixture of both, demonstrating that the antigenic specificity was due to the nucleoside unit as a whole. More interestingly, we could show that the anti-uridine antibodies obtained in this way react with *E. coli* RNA and with polyadenylic acid. Anti-uridine antibodies precipitated with denatured, single chain DNA, but did not react with native DNA.

It thus seems that it is rather easy to obtain antibodies of almost any specificity desired, provided one builds the correct immunogenic molecule. To give another example: antibodies to enzymes have been investigated thoroughly for many years. Now, what about antibodies to coenzymes and vitamins? By using similar approaches we have prepared a *pyridoxyl polymer* (Fig. 6) which led to antibodies reacting with pyridoxal [34]. These anti-pyridoxal antibodies inhibited the enzymatic activity of glutamic-oxaloacetic transaminase. Similarly, antibodies against folic acid and against methotrexate were obtained [35].

It is possible also to prepare synthetic antigens containing a glycolipid, such as cytolipin H, which lead to antibodies recognizing both the sugar moiety and the lipid moiety [36].

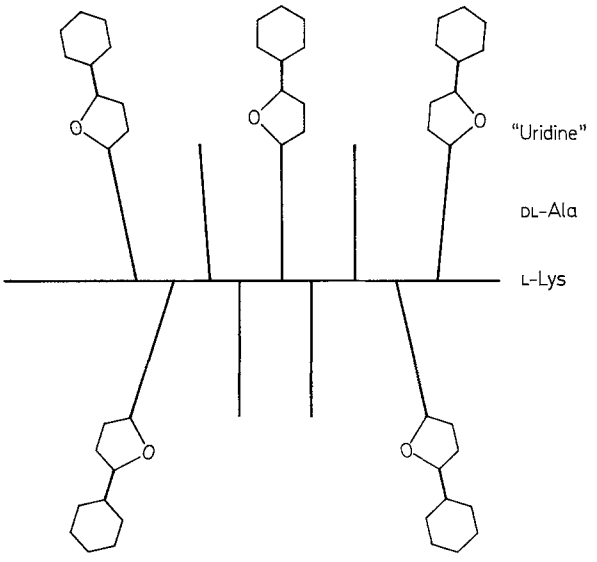

Fig. 5. Schematic representation of the multichain nucleoside-polypeptide conjugate uridyl-multi-poly-DL-alanyl—poly-L-lysine

Genetic Control

Until now we have discussed mainly the use of the synthetic approach in our efforts to understand better the molecular basis of antigenicity. A similar approach has been found very useful also in the elucidation of other immunological phenomena, such as delayed hypersensitivity, immunological tolerance, and the intracellular fate of antigens [2, 14, 37]. I would like to dwell for a moment on the *genetic control of the immunological response*, an area which led recently to many interesting results, mainly due to the availability of synthetic antigens. I shall give just one example from our work with H. O. MCDEVITT [12, 28]. Immunization of two different inbred mouse strains with the synthetic branched antigen in which peptides containing tyrosine and glutamic acid were attached to branched polyalanine, led to a very good antibody production in one strain and to a very bad antibody production in the other strain. This capacity to respond well or badly to the antigen is genetically controlled as is clear from cross-breeding experiments between the two strains.

The interesting result which I want to point out is that, when a molecule is built which has an identical size and shape, and an almost identical composition, with the exception that tyrosine is replaced with histidine, the immune response in these two genetically different strains of mice is reversed. Now, the CBA mice are good responders, and the C 57 do not respond at all. If tyrosine is replaced with phenylalanine, both strains are equally good responders. So, we deal here with a determinant-specific genetic control of the immune response. In other words, while our immunity may not be inherited, our capacity to be immunized against a specific antigenic determinant may very well be an inheritable trait.

There is not much difference between the response of the two mouse strains toward protein antigens. This

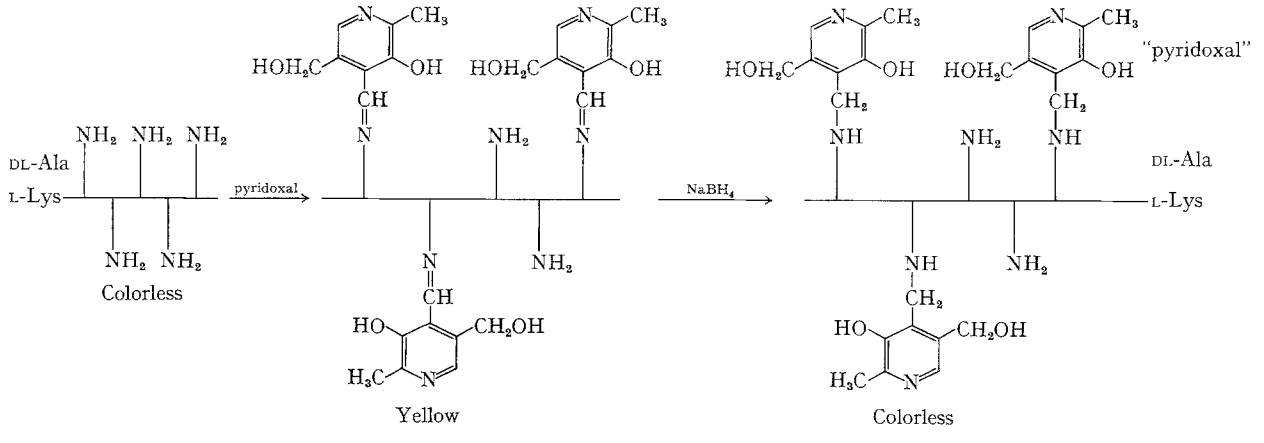

Fig. 6. Schematic representation of the synthesis of multichain pyridoxyl-polypeptide conjugate. From UNGAR-WARON and SELA [34]

is probably due to the complexity of such multideterminant antigens. On the other hand, synthetic antigens may be good models for the study of the genetic control of the immune response as they possess either one specificity determinant, or a very limited number.

Concluding Remarks

In conclusion, the use of synthetic antigens and their conjugates has proved to be a valuable tool in the study of the molecular basis of immunological phenomena. The relative simplicity of these antigenic models facilitates the interpretation of the results obtained with them, and sometimes permits the detection of effects, such as genetic variations, which are not easily observable with complex natural antigens. Although studies of antibodies to synthetic antigens are only in the initial stages, they should prove useful in the elucidation of antibody structure and biosynthesis [39, 40]. 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 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.

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Natrium-22-Ablagerung durch Regen im Jahr 1968

H. MÜH und A. SITTKUS

Max-Planck-Institut für Kernphysik, Heidelberg;
Außenstelle Freiburg-Schauinsland

Seit Januar 1968 wurde die Na-22-Ablagerung durch Regen in Freiburg (47,9° N, 7,8° O) beobachtet. Das Sammeln der Niederschläge, die Aufbereitung der Proben und der Nachweis geschah ähnlich wie bei RÖDEL [1].
In Tabelle 1 ist die monatliche Ablagerung des Na-22 eingetragen. Die Abscheidung läßt den erwarteten Jahresgang, der mit der jahreszeitlichen Variation des Austausches zwischen Stratosphäre und Troposphäre erklärt wird, gut erkennen.
Die Ablagerung beträgt 1968 etwa $7{,}5 \times 10^7$ Atome/m² und Jahr. Sie ist gegenüber dem Wert von RÖDEL [2] für 1966 wieder deutlich angestiegen (Tabelle 2) und übersteigt die Produktion durch die kosmische Ultrastrahlung ($2{,}7 \times 10^7$ Atome/m² × Jahr in 50° Breite [3]) um $5{,}8 \times 10^7$ Atome/m² × Jahr. Der Überschuß ist mit großer Sicherheit der Produktion bei den *chinesischen Kernwaffenversuchen*, besonders der H-Bombenerprobung vom Juni 1967, zuzuschreiben.

Tabelle 1. *Monatliche Ablagerung von Na-22 durch Regen*

1968	10^6 Atome/m²
Januar	2,10
Februar	4,50
März	1,15
April	12,05
Mai	17,90
Juni	9,05
Juli	9,60
August	5,95
September	4,15
Oktober	1,90
November	4,10

Tabelle 2. *Jährliche Na-22-Ablagerung*

Jahr	Ort	10^7 Atome/m² × Jahr
1963	Heidelberg	50,5
1964		26,8
1965		8,0
1966		4,1
1968 (Jan.—Nov.)	Freiburg	7,3

Eingegangen am 5. und 11. Februar 1969

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