Handbook of Experimental Immunology

EDITED BY

D. M. WEIR MD

THIRD EDITION

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Preface

Experimental immunology has continued to grow exponentially since the last edition of this text and this is reflected in the new material and increased number of contributors to this new edition. Like the previous edition the material in the 3rd Edition has been grouped into the three main subdivisions of experimental immunology. The chapters in each subdivision have been brought together into sections. Thus in Volume 1, Immunochemistry, there are sections on antigens, complement, immunoglobulin purification and characterization, immunogenetics and antigen–antibody interactions. In Volume 2, Cellular Immunology, under the heading of lymphocytes, there are chapters on a large variety of procedures for the preparation, separation, analysis, preparation of hybrids and demonstration of activities of lymphocytes. Similarly there is a section on phagocytes and a section on cellular antigens and products.

Volume 3, Application of Immunological Methods, contains chapters on immunological methods in bacteriology, virology, mycology, protozoology and helminthology. There are chapters on mediators and antibodies in hypersensitivity reactions and a chapter on tests of immune function. The appendices contain discussion of statistical aspects of planning and design of immunological experiments statistical analysis of data, adjuvants and immunization, laboratory animal techniques and mouse-breeding procedures.

There has been extensive revision of the majority of chapters and some of the material from earlier editions has been deleted. There are many new chapters on methods that have come into use since the last edition.

This book is directed primarily at research workers in each of the three subdivisions of immunology which cover a wide range of scientific disciplines. Workers in the field tend to specialize within a limited area, so that the availability in this text of discussion and description of methodology covering each of the main subdivisions should be particularly valuable. Techniques outside the normal expertise of most experimental immunologists are thus made accessible. The inclusion of appendices on experimental design and statistical analysis of immunological data should also be convenient and useful aids in the planning of experiments and analysis of data.

I should like to thank the many contributors for their efforts and co-operation that have made it possible to bring together information on a very wide range of immunological topics and for their help in trying to fulfil the requirements for a uniform format for the presentation of the material.

Thanks are due to the many colleagues who have offered constructive advice arising from the use of earlier editions. Dr Len Herzenberg has encouraged us to prepare supplements to the text as important new procedures are described. It should thus be possible for the reader to gain access to new material within a short time of its original description without having to wait for a new edition of the complete text. Contributors and readers are encouraged to inform the editor of interesting new developments in their field.

The index was prepared by Mr S. Revett and the sub-editing carried out by Linda Adler and Rosemary Walton. Dr Caroline Blackwell provided editorial advice on microbiological aspects of the text. I should like to acknowledge the invaluable and highly efficient support provided by the publishers, particularly Mr Per Saugman and Mr Nigel Palmer and his staff of the Edinburgh office.

D. M. Weir

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CHAPTER 1

Preparation of synthetic antigens

M. SELA & SARA FUCHS

The notion of antigen encompasses two distinct properties, namely its immunogenicity and antigenic specificity. *Immunogenicity* is the capacity to provoke an immune response and is independent of the specificity of the antibodies formed. *Antigenic specificity* is reflected in the nature of the antibody combining site and is defined as the capacity of a molecule, or small portions of it, to react with antibodies, already formed.

One approach for studying these two properties on a molecular level was to start with a protein which by itself is a very poor immunogen, and to enhance its immunogenicity by modifying it chemically. Indeed, attaching peptide chains of tyrosine to gelatin (Fig. 1.1) enhances the immunogenicity [1]. However, the antigenic specificity of the antibodies obtained depended on the amount of tyrosine attached. As little as 2 per cent tyrosine sufficed to enhance strongly the antigenicity of gelatin. The antibodies formed cross-reacted well with gelatin, suggesting that although tyrosine makes the molecule a better immunogen, it does not change the specificity very much [2]. On the other hand, attachment of 10 per cent of tyrosine to gelatin converted it again into a very good immunogen, but this time the specificity was changed, and 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 nonimmunogenic. Our choice was a multichain polymer of DL-alanine [3]. This branched polymer (Fig. 1.2) 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. When we attached some tyrosine, or tyrosine and glutamic acid, to this non-immunogenic material and injected the compounds obtained into rabbits, the antisera formed contained antibodies to these materials, and these antibodies were highly specific [4, 5]. Thus, we had for the first time a synthetic polymer which was a potent and specific antigen. Independently Gill and Doty [6] as well as Maurer [7] have shown that some linear synthetic polypeptides composed exclusively of α-amino acids are immunogenic.

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 [8, 9].

In detailed studies concerned with the elucidation of the molecular basis of antigenicity it was shown that linear as well as branched synthetic polypeptides may be potent and specific antigens [7, 8].

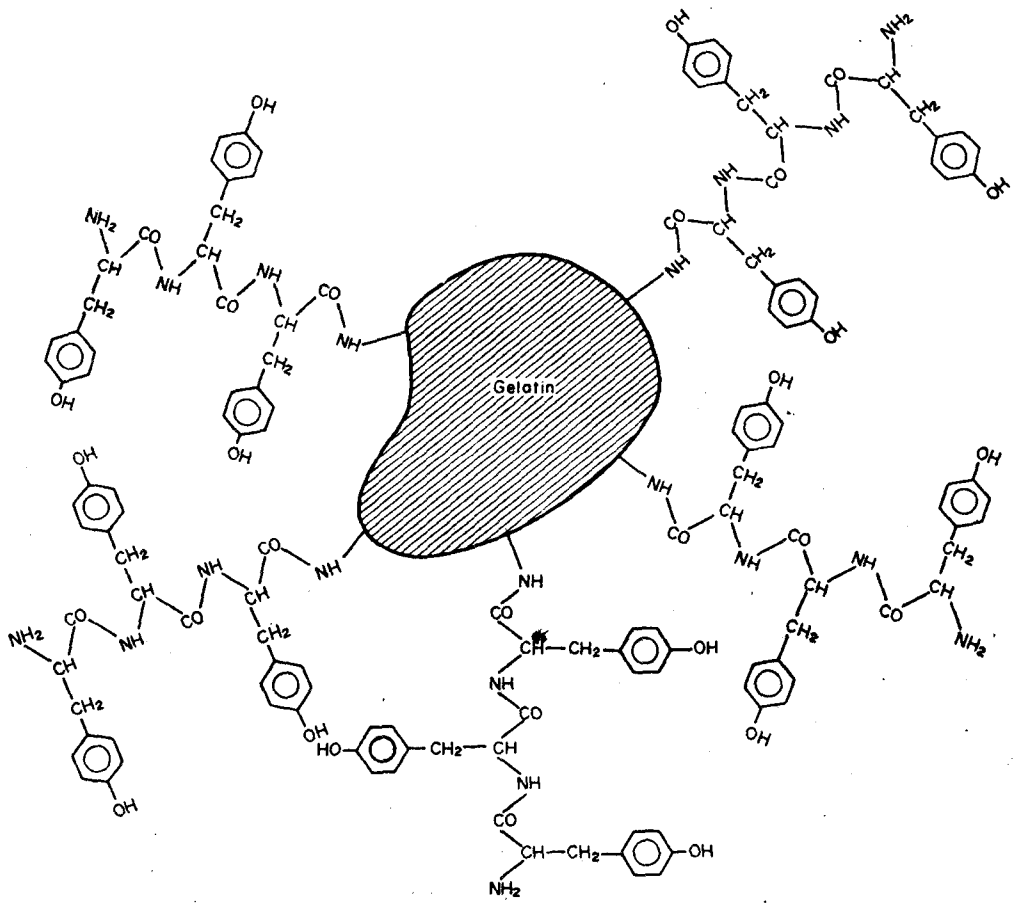


FIG. 1.1. A schematic description of polytyrosyl gelatin which was prepared by the attachment of peptide chains of tyrosine to gelatin.

It seems that a certain complexity (namely participation of several different amino acid residues in the synthetic molecule), which may reflect both the variations in composition as well as in conformation, is necessary for immunogenicity. 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 [5]. The presence of electrical charges on a macromolecule is not a minimum requirement for it to be immunogenic as completely nonionizable polypeptide was shown [10] to be immunogenic. It has also been found that the immunogenicity is better when both negative and positive charges are present than when only one kind of charge appears on a molecule [11]. The molecular weight requirements for an immunogenic molecule are not very critical as small oligopeptides, provided they possess the appropriate composition, may be immunogenic as well [12–14].

Although polypeptides composed of D-amino acids are poor immunogens, they can provoke the production of specific antibodies when inoculated in very low doses [9]. It is probably not the optical configuration but rather the catabolism rate of such polymers which is important for efficient immunogenicity, as polymers prepared mostly from L-amino acids, but in such a way that their enzymic cleavage is prevented, behave similarly to D-amino acid polymers [15, 16]. The spatial conformation of

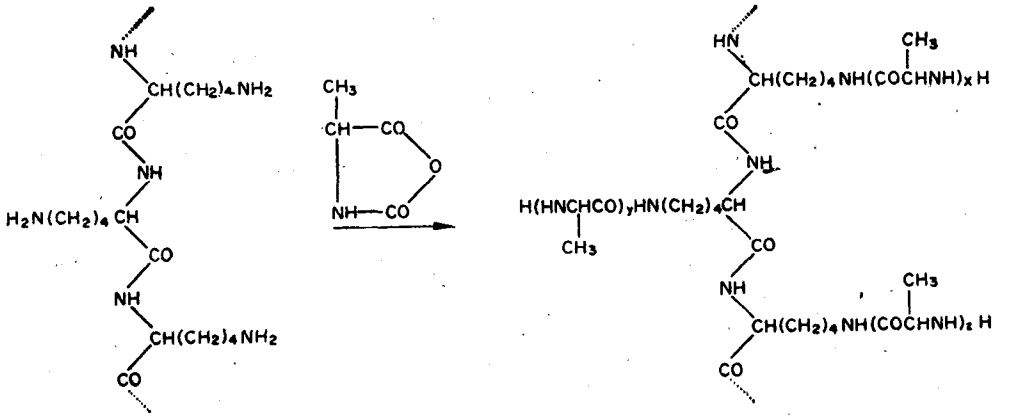


FIG. 1.2. A schematic description of the preparation of multichain poly-DL-alanine from *N*-carboxy-DL-alanine anhydride and poly-L-lysine.

the antigenic determinants in the immunogenic macromolecule is critical in determining the specificity of the antibody formed. This has been demonstrated with both synthetic antigens possessing, respectively, sequential and conformational determinants [17, 18] and with protein antigens. In the latter case this has been exemplified with a fragment of lysozyme which had been synthesized and converted into an immunogen by attachment to multichain poly-DL-alanine [19]. These observations, as well as others described above, suggest that recognition of the antigenic determinant occurs while the immunogenic macromolecule is still intact [9].

The synthetic antigens are appropriate models for natural antigens [8, 9] as they manifest the same immunological phenomena such as, e.g. immunological tolerance, antigenic competition, a variety of *in vitro* reactions, delayed hypersensitivity [20], and fate and metabolism of an antigen [21]. The synthetic antigens were shown to be immunogenic, in rabbits, guinea-pigs, mice, rats, goats, sheep, monkeys and humans. They are immunogenic even at very low doses (microgram quantities) [22].

Among the manifold uses of synthetic antigens in the elucidation of immunological phenomena special mention should be made of the investigations of the size and nature of combining sites of antibodies complementary to peptides [e.g. 23], of the inverse relationship between the net electric charge of an antigen and its respective antibody [24, 25], and of the precise description of the genetic control of immune response [26]. In the last case the simplicity of the antigenic models permitted the discovery of determinant-specific genetic control [27], of the linkage of the genetic control to the main histocompatibility antigen [28], as well as the genetic control of immune responsiveness toward unique determinants [29, 30]. Cellular studies have led, *inter alia*, to primary antibody response *in vitro* [31], to the role of various cell types in the net charge relationship [32], as well as in the genetic control of the immune response [33].

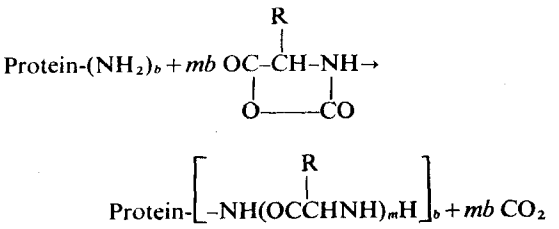
Starting from synthetic polypeptides, immunogens may be prepared which are capable of provoking antibodies of almost any specificity desired. Immunogens have been synthesized by attaching to polypeptides substances such as sugars, nucleosides, pyridoxal, the glycopeptide cytolipin H, the pharmacologically active peptides angiotensin and bradykinin [9], ferrocene [34] and digoxin [35].

Polypeptidyl proteins

Polypeptidyl proteins are of interest in immunological studies, as peptidylation of proteins gives rise to antigens with new and well-characterized specificity. Peptidylation may in some cases also result in increase or decrease of immunogenicity. From the point of view of their immunological properties, the attached peptides may be considered as haptens composed of amino acids. By peptidylation it is possible to enrich a protein with any amino acid desired. Immunization with polypeptidyl proteins may lead to the production of antibodies

directed against several different determinants, namely antibodies to determinants on the protein molecule, to the attached peptides and also to determinants containing both a part of the protein and a part of the polypeptide.

Polypeptidyl proteins usually have been prepared by the polymerization of *N*-carboxy-α-amino acid anhydrides in aqueous media using proteins as multifunctional initiators. *N*-Carboxy-α-amino acid anhydrides are prepared from the respective amino acids and phosgene [36]. The peptidylation reaction was first described by Stahmann and Becker [37, 38]. The α-amino and ε-amino groups of the protein were found to initiate the polymerization leading to a polypeptidyl protein according to the following scheme:



where m is the average chain length and b is the number of chains per molecule of protein. The polymerization was found to proceed under mild conditions (aqueous media, low temperature and almost neutral pH), which do not cause denaturation of most proteins.

Peptidyl proteins may be prepared also by reaction of preformed peptides with proteins. In this case either the peptide is activated before the reaction, e.g. a series of $(Ala)_nGly$ was attached to ribonuclease and to rabbit serum albumin using the active succinimide esters [23], or a cross-linking reagent such as carbodiimide is added, e.g. the attachment of bradykinin to rabbit serum albumin [39].

Methods

Preparation of poly-L-alanyl human serum albumin

Poly-L-alanyl human serum albumin is prepared from *N*-carboxy-L-alanine anhydride and human serum albumin [40]. The *N*-carboxy-L-alanine anhydride is prepared in the following way: L-alanine (10 g) and anhydrous dioxane (200 ml) are placed in a two-necked, 1-litre, round-bottomed flask, equipped with a reflux condenser, a gas inlet tube extending below the surface of the reaction mixture, and a magnetic stirring bar. The mixture is kept at 50° C in a water bath, and a stream of phosgene (withdrawn from a cylinder and dried by bubbling through concentrated sulphuric acid) is passed through the gas inlet for 3 hours, whereupon all the solid goes into solution. The hydrogen chloride formed and the excess of phosgene are passed through the top of the condenser into a wash bottle containing aqueous ammonia. After 3 hours the supply of phosgene is stopped, and a stream of dry nitrogen is blown through the solution for 2 hours, to remove excess phosgene. The solution is filtered through glass wool, and the clear solution is concentrated *in vacuo* at 40° C. The oily residue is dissolved in anhydrous ethyl acetate (100 ml), which is removed by distilling *in vacuo*. This procedure is repeated once, and the residue is dissolved in about 20 ml of anhydrous petroleum ether, and stored in the deep-freeze until it crystallizes. If the oil does not crystallize, the solvent is removed by distillation *in vacuo*, and the treatment with ethyl acetate is repeated. The crystals are collected, washed with petroleum ether, and dried *in vacuo* over phosphorus pentoxide. *N*-Carboxyanhydrides of several amino acids are available commercially.

For the preparation of poly-L-alanyl human serum albumin 1 g of human serum albumin (HSA) is dissolved in 80 ml of 0·05 mol/l phosphate buffer, pH 7·0 (in a 500 ml Erlenmeyer flask). The flask is cooled to about 4° C, and a solution of 0·8 g of *N*-carboxy-L-alanine anhydride in 40 ml of anhydrous dioxane is added to the protein solution. (In case the anhydride is not completely soluble in the dioxane, the mixture is filtered quickly through a filter paper.) When the protein and anhydride solutions are mixed, the polymerization takes place with the evolution of carbon dioxide. The reaction mixture is left in the cold room overnight, and then dialysed against several changes of distilled water. The contents of the dialysis bag are brought to pH 7·0 with a few drops of 1·0 mol/l sodium hydroxide, centrifuged at 8000 rev/min to remove precipitates, and the clear supernatant fluid is lyophilized.

The enrichment in alanine is calculated from amino acid analyses of hydrolysates before and after peptidylation. The number of alanine chains attached per protein molecule is quantitated by dinitrophenylation or by deamination [41]. The average chain-length of the peptides attached may thus be calculated.

Peptidylation of other proteins with different

amino acid peptides may be achieved in a similar way. In case the *N*-carboxyamino acid anhydride is blocked (e.g. *N*-carboxyanhydride of γ-benzyl glutamate or of ε,*N*-carbobenzoxylysine), the blocking groups are removed after the peptidylation reaction.

Linear polymers

Many linear polymers of different amino acid compositions and molecular sizes have been used for immunological studies [8]. As homopolymers were shown to be essentially non-immunogenic, a variety of *copolymers* composed of two, three, four or more different amino acid residues were prepared and tested as immunogens in several laboratories. Most of the copolymers studied were prepared by *random* copolymerization of the respective *N*-carboxy-α-amino acid anhydrides in the appropriate residue molar ratio. Such polymerization gives rise usually to copolymers which do not possess a unique amino acid sequence or conformation. Another approach which has been in use in recent years is to study immunological properties of *ordered* polymers which do possess repeating amino acid sequences, and, in some cases, defined unique conformations. Such polymers are obtained by polymerization of short peptides and are thus composed of unique repeating sequences. Immunological studies of ordered polymers are of special interest since the antigenic determinants of these immunogens are controlled by the three-dimensional conformation of the molecule rather than directly by amino acid sequences, as is the case of random polymers. An ordered polymer, poly(L-Pro–Gly–L-Pro) obtained by polymerization of the tripeptide L-Pro-Gly-L-Pro which has several physicochemical properties similar to those of collagen [42], was shown to be immunogenic in several species [43], and cross-reactions were observed with several natural collagens [43, 44]. However, no cross-reactions with collagen were detected after immunization with a random copolymer poly(L-Pro^{66}, Gly^{34}), of amino acid composition similar to that of the ordered polymers, but lacking the unique collagen structure. In a similar study it was shown that antibodies to the ordered polymer poly(L-Tyr-L-Ala-L-Glu) [17] are directed to conformational antigenic determinants, whereas antibodies to the random poly (Tyr^{33}, Ala^{33}, Glu^{33}) are directed to sequential antigenic determinants.

Methods

Preparation of a random copolymer poly(L-Tyr^{33}, L-Ala^{34}, L-Glu^{33})

N-Carboxyanhydrides of γ-benzyl L-glutamate and of L-tyrosine are prepared from phosgene and, respectively, γ-benzyl-L-glutamate and L-tyrosine, in a similar way to the preparation of *N*-carboxy-L-alanine anhydride described above. *N*-Carboxy-L-tyrosine anhydride is crystallized from a boiling dioxane solution. In a 0·5 litre flask equipped with a calcium chloride drying tube (or a nanometer to test for evolution of gas) and a magnetic stirring bar, the following anhydride solutions are mixed: 2·07 g (1 mmol) *N*-carboxy-L-tyrosine anhydride dissolved in 40 ml of anhydrous dioxane (dissolved by heating the dioxane solution to about 70° C), 1·15 g (1 mmol) *N*-carboxy-L-alanine anhydride in 20 ml of anhydrous dioxane and 2·63 g (1 mmol) *N*-carboxy-γ-benzyl-L-glutamate anhydride in 40 ml of anhydrous dioxane. Triethylamine (0·042 ml) is added immediately to initiate the polymerization, and the reaction mixture is left overnight with stirring at room temperature. After this period the polymer is precipitated with ether (about 500 ml). The precipitate is filtered and washed several times with ether, dried *in vacuo* over sulphuric acid and then over phosphorus pentoxide.

To remove the protecting groups (in this case the γ-benzyl groups of the glutamic acid residues), the polymer is treated in a glass-stoppered vessel with 100 ml of a 33 per cent solution of hydrogen bromide in glacial acetic acid for 72 hours in the cold room. After that time precipitation of the copolymer is completed with anhydrous ether, the copolymer is collected on a sintered glass filter, washed several times with ether, and dried *in vacuo*. The polymer is dissolved in 0·05 mol/l sodium hydroxide, dialysed against several changes of distilled water and lyophilized.

Preparation of an ordered copolymer poly(L-Tyr-L-Ala-L-Glu)

The ordered polymer poly(Tyr-Ala-Glu) is prepared according to the procedure described by Ramachandran *et al.* [45]. This polytripeptide is obtained by the poly condensation of the *N*-hydroxysuccinimide ester of *O*-benzyl-tyrosyl-alanyl-γ-benzyl glutamic acid in dimethylformamide (DMF), and removal of the protecting groups with anhydrous hydrogen bromide in glacial acetic acid. The active ester is obtained by the reaction of *N*-blocked tri-

peptide with *N*-hydroxy-succinimide in the presence of dicyclohexylcarbodiimide (DCC) in the following procedure: *N*-tert-butyloxycarbonyl-*O*-benzyl-tyrosyl-alanyl-γ-benzyl glutamate (6·62 g, 10 mmol) and *N*-hydroxysuccinimide (1·15 g, 10 mmol) are dissolved in 60 ml of a mixture of dioxane and ethyl acetate (3:1) and cooled to 0° C. DCC (2·06 g, 10 mmol) is added and the reaction mixture stirred at 0° C for 1 hour and then at 4° C for 20 hours. Dicyclohexylurea is filtered off and washed with ethyl acetate. The filtrate and washings are combined and the solvents removed *in vacuo*. The residue is treated with a drop of glacial acetic acid and then dissolved in 40 ml warm isopropanol. The solution is filtered from traces of insoluble material and allowed to attain room temperature slowly. The product crystallizes readily and is filtered after standing for 20 hours and then the crystals are washed with ice-cold isopropanol and absolute ether.

The ester obtained (6 g, 8 mmoles) is dissolved in 50 ml 2 N hydrochloric acid in dioxane and kept at room temperature for 2 hours. The solvent is removed *in vacuo*, the residue is washed several times with absolute ether and then filtered and dried over phosphorus pentoxide and sodium hydroxide *in vacuo*. The tripeptide active ester hydrochloride thus obtained is dissolved in 18 ml dry DMF. Triethylamine (1·4 ml, 10 mmol) is added. Triethylamine hydrochloride crystallizes out immediately and is quickly filtered off (any moisture should be avoided), and the crystals are washed with DMF. The filtrate and washings (about 25 ml) are collected in a 100 ml round-bottomed flask fitted with a calcium chloride tube. The polymerization mixture is stirred by rotating the flask. The solution becomes very viscous within 10 min and it is progressively diluted by adding 2 ml DMF every 24 hours. The polymerization is allowed to proceed for 6 days. The viscous solution is then poured into 300 ml cold water and stirred at room temperature for 2 hours. The precipitate is allowed to stand for 12 hours at 4 °C and filtered and washed with water. The polymer is dried over phosphorus pentoxide *in vacuo*. This polymer is soluble only in trifluoroacetic acid.

To remove the protecting groups the polymer (4 g) is dissolved in 10 ml anhydrous trifluoroacetic acid, and then 30 ml of 45 per cent HBr in glacial acetic acid is added. The mixture is kept at 4° C for 72 hours. The product is isolated by precipitating into 500 ml absolute ether. The precipitate is stirred vigorously with several portions of ether and ethyl acetate, and the product is filtered and dried over sodium hydroxide *in vacuo*. The deblocked polymer, which is soluble at pH values above 5·5, can be fractionated on a Sephadex G-50 column into several fractions of different degrees of polymerization. Polymers with molecular weights in the range of 60 000 have been obtained by this method.

Multichain polymers

A multichain polymer is a branched polymer in which linear polymeric chains are attached to a polyfunctional core. Multichain polyamino acids may be obtained by the initiation of the polymerization of *N*-carboxy-α-amino acid anhydrides with multifunctional initiators. Both proteins and synthetic polypeptides containing numerous free amino groups have been used as multifunctional initiators. Polymerization of *N*-carboxy amino acid anhydrides on proteins leads to polypeptidyl proteins, as described above, whereas polymerization on synthetic polypeptides (e.g. polylysine, polyornithine or copolymers containing these basic amino acids) leads to synthetic multichain (or branched) polyamino acids [3]. In addition to these multichain polymers which are prepared by *random* polymerization of *N*-carboxy-α-amino acid anhydrides with multifunctional initiators, *ordered* multichain polymers have been recently prepared by the attachment of synthetic oligopeptides (e.g. tetrapeptides) to multifunctional initiators. Such ordered multichain polypeptides proved a useful tool for further detailed analysis of the genetic control of immune response to synthetic polypeptides [46, 47].

Because of the relative proximity of their polypeptide side chains, their relatively low viscosities, and high sedimentation coefficients, multichain polyamino acids may perhaps serve as better models for globular proteins than linear polyamino acids. As they are also relatively easily prepared, and the groups at the periphery of the molecule may be easily changed, multichain synthetic polypeptides have been used extensively in different immunological studies [8, 9].

Methods

Preparation of multi-copoly(L-*phenylalanyl*, L-*glutamyl*)*poly*-DL-*alanyl*-*poly*-L-*lysine* (Fig. 1.3) [48]

The *N*-carboxyanhydrides of the amino acids that compose this polymer are prepared by reacting the respective amino acid (or blocked amino acid)

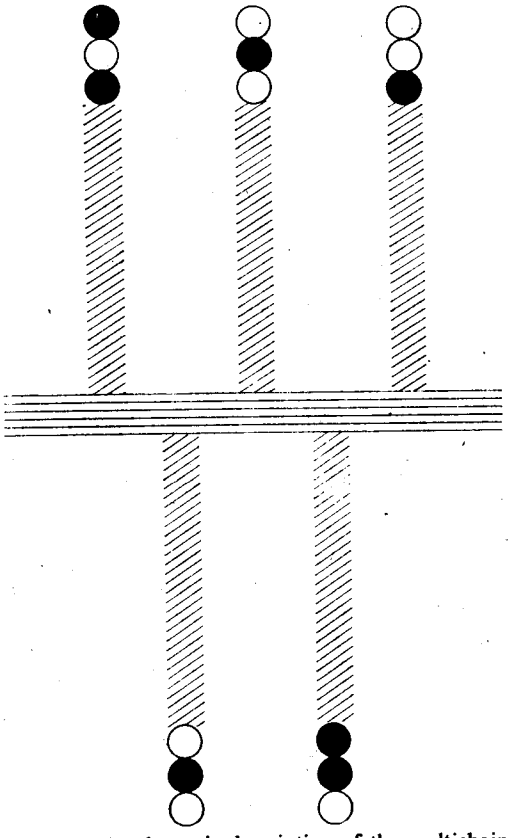


FIG. 1.3. A schematic description of the multichain copolymer poly(Phe,Glu)-poly(DLAla)--poly(Lys) (designated as (Phe,G)-A--L) in which L-phenylalanine and L-glutamic acid residues are attached to multichain poly-DL-alanine; horizontal lines, poly-L-lysine; diagonal hatching, poly-DL-alanine; closed circles, L-phenylalanine; open circles, L-glutamic acid.

with phosgene in a similar way to the preparation of *N*-carboxy-L-alanine anhydride described above.

The first step is the preparation of multichain poly-DL-alanine (multi-poly-DL-alanyl--poly-L-lysine). The reason for using DL-alanine rather than L-alanine is because poly-L-alanine as well as poly-D-alanine are utterly insoluble in water, in contrast to poly-DL-alanine which is water-soluble. Multichain poly-DL-alanine is prepared from *N*-carboxy-DL-alanine anhydride and poly-L-lysine as exemplified in Fig. 1.2 in the following way: a solution of 1 g of poly-L-lysine hydrobromide [36] in 300 ml of 0·05 mol/l phosphate buffer, pH 7·0, is introduced into a 1 litre flask. The flask is cooled with ice to about 2° C, and a solution of 14 g of *N*-carboxy-DL-alanine anhydride in 200 ml of anhydrous dioxane is added with vigorous shaking. At this stage there is a strong evolution of carbon dioxide. The reaction mixture is left with magnetic stirring in the cold room overnight and then dialysed against several changes of distilled water and lyophilized.

Multi-poly(L-phenylalanyl,L-glutamyl)-poly-DL-alanyl--poly-L-lysine, which is described in a schematic way in Fig. 1.3, and designated as (Phe,G)-A--L, is prepared by copolymerization of *N*-carboxy-anhydrides of L-phenylalanine and γ-benzyl-L-glutamate on multichain poly-DL-alanine as a multifunctional initiator: a solution of 6 g of multichain poly-DL-alanine in 500 ml of 0·05 mol/l phosphate buffer, pH 7·0, is introduced into a 2 litre flask. The flask is cooled with ice to about 2° C, and a solution of 3·9 g (15 mmol) of *N*-carboxy-γ-benzyl-L-glutamate anhydride and 1·9 g (10 mmol) of *N*-carboxy-L-phenylalanine anhydride in 120 ml of anhydrous dioxane (each anhydride is dissolved separately in dioxane and the two anhydride solutions are mixed before the addition to the aqueous solution) is added with vigorous shaking. There is an evolution of carbon dioxide, and a precipitate is formed. The reaction mixture is left under stirring in the cold room overnight. The precipitation of the reaction product is completed by adding 3 volumes of cold acetone. (It is desirable to determine on a small sample how many volumes of acetone give an easily filterable precipitate). The mixture is allowed to stand for several hours in the cold, the supernatant is decanted and the precipitate is centrifuged or filtered by suction, washed with acetone and water, and dried over sulphuric acid and phosphorus pentoxide *in vacuo*. The protecting benzyl groups are removed as described above for the linear polymer. The final product is dissolved in water (or alkaline solution), dialysed against several changes of distilled water and lyophilized. The amino acid composition of the multichain polymer is determined from amino acid analysis and its molecular weight from sedimentation and diffusion in the ultracentrifuge [5]. In some cases the polymer may be chromatographed on a Sephadex (G-150) column to remove any low molecular weight substances present.

Preparation of multi-copoly(L-tyrosyl, L-glutamyl)-poly-L-prolyl--poly-L-lysine [15]

This multichain copolymer, designated as (T, G)-Pro--L, is prepared by polymerization of *N*-carboxy

anhydrides of L-tyrosine and γ-benzyl-L-glutamate on multichain poly-L-proline which serves as the multifunctional initiator. Multichain poly-L-proline is prepared in dimethyl sulphoxide from polylysine and *N*-carboxy-L-proline anhydride in the following way: poly-L-lysine hydrobromide (0·7 g) is dissolved in 200 ml of dimethyl sulphoxide. 0·5 ml triethylamine is added. *N*-Carboxy-L-proline anhydride (14 g, prepared from L-proline and phosgene [49], and used immediately after preparation since this particular anhydride is unstable), dissolved in 100 ml of dimethyl sulphoxide is then added to the reaction mixture, under vigorous stirring at room temperature. Carbon dioxide evolution starts immediately and the polymerization is allowed to proceed for 24 hours with stirring at room temperature. The reaction mixture which is opalescent and very viscous after 24 hours, is exhaustively dialysed against distilled water to remove dimethyl sulphoxide. The gelatinous precipitate formed during the dialysis is concentrated by flash evaporation, freeze dried, and subsequently dissolved in 300 ml of anhydrous formic acid at 25° C. This treatment brings about the conversion of the water-insoluble polymeric side chains (of polyproline I form) into the water-soluble polyproline II form. The resulting formic acid solution is kept for 1 hour at 25° C, and then dialysed against several changes of distilled water. The contents of the dialysis bags are concentrated by flash evaporation, lyophilized, and stored at −20° C prior to use.

The preparation of (T,G)-Pro--L from multichain poly-L-proline and the *N*-carboxy anhydrides of L-tyrosine and γ-benzyl-L-glutamate is performed similarly to (Phe,Glu)-A--L. The terminal *imino* groups of polyprolyl side chains in the multichain polyproline serve as initiators in this polymer, whereas in polyalanine the initiation occurs at terminal *amino* groups. It seems that more of the *N*-carboxy anhydrides are needed to achieve the same enrichment in the polyproline system than in the polyalanine system. In a typical preparation of multi-poly(L-tyrosyl,L-glutamyl)-poly-L-prolyl--poly-L-lysine, multichain poly-L-proline (1·5 g) is dissolved in 150 ml of 0·05 mol/l phosphate buffer, pH 7·0 in a 1 litre flask. The flask is cooled with ice to about 2° C, and a solution of 4·2 g of *N*-carboxy-γ-benzyl-L-glutamate anhydride and 3·0 g of *N*-carboxy-L-tyrosine anhydride in 120 ml of anhydrous dioxane (each anhydride is dissolved separately in dioxane and the two anhydride solutions are mixed before the addition to the aqueous solution; *N*-carboxy-L-tyrosine anhydride is dissolved by heating the dioxane solution to about 70° C) is added with vigorous shaking. The polymerization, precipitation of the polymer and removing the blocking groups are performed as described above for (Phe,Glu)-A--L.

Preparation of the ordered multichain polypeptide (L*Tyr*-L*Tyr*-L*Glu*-L*Glu*)-*poly*(DL*Ala*)--*poly*(L*Lys*) [47] This multichain ordered polymer, designated as (T-T-G-G)-A--L is prepared by conjugation of the tetrapeptide LTyr-LTyr-LGlu-LGlu with multichain poly-DL-alanine. The dipeptides and tetrapeptide are synthesized using a procedure analogous to that of Ramachandran, Berger and Katchalski [45]. The tetrapeptide is prepared from two dipeptides, one of which is blocked at the α-amino-terminus with the tert-butyloxycarbonyl group and activated at the α-carboxyl terminus in the form of *N*-hydroxysuccinimide ester. The second peptide is free at both ends. The dipeptides are synthesized using the same procedure [45] from the appropriate amino acids.

A schematic description of the various steps in the synthesis of (T-T-G-G)-A--L is given in Fig. 1.4.

N-tert-Butyloxycarbonyl-*O*-benzyltyrosine hydroxysuccinimide ester (I): *N*-tert-butyloxycarbonyl-*O*-benzyltyrosine (3·7 g, 10 mmol) and *N*-hydroxysuccinimide (NHSuI, 1·15 g, 10 mmol) were dissolved in 60 ml solution of dioxane:ethyl acetate (3 : 1) and cooled to 0° C. Dicyclohexylcarbodiimide (DCC, 2·06 g, 10 mmol) was added and the mixture was stirred for 30 min at 0° C and then left for 20 hours at 4° C. Dicyclohexylurea (DCU) was filtered off, the filtrate concentrated *in vacuo* to oil, and 20 ml of warm isopropanol were added to the residue. The solution was cooled slowly to room temperature. The product crystallized readily and was collected after 20 hours at 4° C. Yield: 3 g, m.p.: 150–151° C.

N-tert-Butyloxycarbonyl-*O*-benzyltyrosyl-*O*-benzyltyrosine (II): *O*-Benzyltyrosine (1·8 g, 6·5 mmol) was suspended in 60 ml water and 1·1 g (13 mmol) sodium bicarbonate was added. Compound I (3 g, 6·5 mmol) was dissolved in 60 ml dioxane and added to the aqueous solution, and the reaction mixture was kept with stirring for 20 hours at room temperature. The dioxane was removed *in vacuo*. The aqueous solution was diluted with 100 ml water and the pH was adjusted to 3 with 10 per cent citric acid. The oily product which separated was extracted into ethyl acetate. The organic phase was washed with water, followed by washes with saturated sodium chloride solution, and then dried over anhydrous sodium sulphate. Most of the solvent was removed,