# Monographs in Virology

Vol. 4

# Viral Structural Components as Immunogens of Prophylactic Value

A. Robert Neurath and Benjamin A. Rubin

# Viral Structural Components as Immunogens of Prophylactic Value

A. Robert Neurath and Benjamin A. Rubin
Wyeth Laboratories, Inc., Department of Research and Development, Philadelphia, Pa.

With 10 figures and 1 color plate



19 71

S. Karger · Basel · München · Paris · London · New York · Sydney

# Acknowledgements

We are grateful for the participation and assistance of our associates: Dr. A. K. FONTES, Dr. M. DOBKIN, Dr. J. T. STASNY, R. W. HARTZELL, F. P. WIENER, H. KIMMEL, S. K. VERNON, W. A. PIERZCHALA, J. SILLAMAN, L. SCHNEIDER, A. WEISSER and R. BLOOM (experimental); J. F. ONGARO (editorial); P. FREIMUTH (audio-visual); T. SCHAFFER (secretarial).

We also gratefully acknowledge the permission by Dr. R. B. COUCH and Dr. G. G. JACKSON to use their data concerning human trials with adenovirus vaccines.

# Index


I. Introduction . . . . . 1
II. General Considerations . . . . . 3
A. Antigenic Sites on Polymeric Proteins and on Their Constituent Monomers 3
B. The Ternary System: Virus-Antibody-Cell . . . . . 6
III. Enlightening Illustrations . . . . . 8
A. Intact Flagella, and Monomeric Flagellin . . . . . 8
B. Bacteriophages Qβ and ΦX174 . . . . . 9
C. Plant Viruses . . . . . 10
D. Hemoglobin and Myoglobin . . . . . 12
E. Alkaline Phosphatase of *Escherichia coli* . . . . . 14
F. Ribonuclease . . . . . 15
G. Foot-and-Mouth Disease Virus (FMDV) . . . . . 15
H. Poliovirus . . . . . 16
I. *Escherichia coli* Phages T2 and T4 . . . . . 19
J. Adenoviruses . . . . . 21
K. Influenza Viruses . . . . . 27
L. Vesicular Stomatitis Virus (VSV) . . . . . 36
IV. Structural Components of Some Other Viruses and Their Ability to Induce Virus-Neutralizing (VN) Antibodies . . . . . 38
A. Vaccinia Virus . . . . . 38
B. Herpesviruses . . . . . 39
C. Measles Virus . . . . . 39
D. Rubella Virus . . . . . 40
E. Arboviruses . . . . . 40
F. Rabies Virus . . . . . 40
V. Interlude . . . . . 42
VI. Experimental Subunit Vaccines: Results of Trials in Humans . . . . . 46
A. Adenovirus Vaccines . . . . . 46
B. Influenza Virus Vaccines . . . . . 49
C. Measles Virus Vaccine . . . . . 52
VII. Recent Developments in Preparing Vaccines from Viral Structural Components . . . . . 54
A. General Considerations . . . . . 54
B. Adenovirus Subunit Vaccines . . . . . 56
C. Influenza Virus Subunit Vaccines . . . . . 65
VIII. Epilogue . . . . . 67
IX. References . . . . . 68

# I. Introduction

Two different observations with two different groups of viruses causing disease in man led to attempts to use viral structural components (subunits) rather than whole virus, either live or rendered noninfectious, for vaccination:

1. Whole influenza viruses caused febrile reactions when injected into man [254, 279] or experimental animals [23]. Soluble envelope antigens obtained by disrupting influenza viruses by diethyl ether or sodium deoxycholate were considerably less pyrogenic than the whole virus and were able to induce virus-neutralizing (VN) antibodies in experimental animals and humans [73, 74, 82, 359].

2. Some human adenoviruses [110, 138, 331] and SV40 virus – a contaminant found in adenovirus and poliovirus vaccines [86, 111] – induced tumors in hamsters and rats [255]. These viruses transformed cells, including human cells, *in vitro* [25]. Part of the SV40 virus genome could be encapsidated by adenoviruses [259].

Despite these observations, the follow-up of persons who had received vaccines contaminated with SV40 or containing live or formaldehyde-inactivated adenoviruses failed to reveal any untoward effects, including tumors, that could be attributed to the vaccines. Nevertheless, the use of adenovirus capsid components as immunizing antigens *(vide infra)* may eliminate any hypothetical risks, if it can be ensured that such antigen preparations are free of genes of viral and cellular origin.

In 1969 influenza vaccines consisting of intact viruses were abandoned in Australia and replaced by subunit vaccines [348]. Thus, it is appropriate to consider the potential usefulness of viral structural components as immunizing antigens of prophylactic value.

This essay is not limited to a discussion of viruses infectious for humans. Section II is devoted to a discussion of complex proteins and their subunits, offering the reader a deeper understanding of the comparative immunologic properties of a polymer and its monomers, and to a short discussion of the ternary system: virus – antibody – cell. Sections III and IV deal with immunogenicity of polymeric proteins and their subunits. Animal and plant viruses,

bacteriophages, enzymes and other proteins are considered. Sections VI and VII are devoted to more basic human needs and comply with the title of this essay. Attention will be given to several human viruses and to results obtained in clinical trials with their structural components. The technology of preparing vaccines consisting of viral structural components will be surveyed and some results from the authors' laboratory will be mentioned. A selected list of references is offered.

This essay should not be considered as a comprehensive listing of past achievements and failures, but as an attempt to unify concepts and to raise questions that might lead to additional experiments and new answers.

# II. General Considerations

## *A. Antigenic Sites on Polymeric Proteins and on Their Constituent Monomers*

The upper limit on the size of antigenic determinants, as estimated from studies on linear polypeptides, is about $30 \times 17 \times 6.5$ Å, or a volume of about 3,000 Å$^3$ (compare with the volume of picornaviruses, which is about $10^7$ Å$^3$) [149]. The size of antigenic sites on proteins is probably of the same magnitude, but the sites may also involve amino acids which are not sequentially arranged, but which are in close proximity due to the folding of the polypeptide chain.

A monomeric protein may contain more than a single antigenic determinant. Because of its limited size, the surface (volume) of the determinant represents only a minor fragment of the surface of the whole protein. The amino acid residues contributing the highest proportion of the binding energy between antigen and antibody are designated as the immunodominant group [149]. In viral capsids and complex proteins (polymers), identical or nonidentical monomeric units interact in a way that involves part of their total surface. Any antigenic determinants occurring in this part of the monomers (viral structural components) are hidden and become available only upon disaggregation of the polymers. Such antigenic determinants are designated cryptotopes [147, 340]. Antigenic determinants which are available at the surface of both the polymers and the monomers are designated metatopes. The proper assembly of identical or nonidentical monomers into polymers may result in the appearance of new antigenic determinants. The determinants may be comprised of amino acid residues from different monomers, or they may arise from conformational changes in the monomers (allosteric transitions) [203]. Such antigenic determinants are designated neotopes.

The immunoinactivation of virus infectivity results from the combination of antibodies with surface antigen(s) on the virus particle [318]. Consequently, metatopes and/or neotopes will play the essential role in eliciting VN antibodies in immunized subjects. If neotopes were absolutely essential or if they played an immunodominant role, antibodies formed as a result of immunization with monomers either would completely fail to neutralize the virus or

would, perhaps, have low avidity (as measured by neutralization of virus infectivity). Considering these problems it should be borne in mind that neotopes may arise not only from the complete assembly of monomers (viral structural components) into a final polymer (viral capsid or envelope), but also from a limited association of a few identical or nonidentical monomers.

Hydrophilic (polar) amino acid side chains usually occur more frequently on the surface of proteins exposed to the surrounding medium than in a position where they are shielded from the medium. The opposite is true for hydrophobic (nonpolar) side chains [163, 242, 251, 253], which may play a decisive role in noncovalent inter-subunit bonds [330]. The presence of hydrophobic side chains of the aromatic α-amino acids in antigenic determinants or in their vicinity increases the rigidity of the antigenic sites. This may result in their enhanced antigenicity [295]. Therefore, it is conceivable that cryptotopes participate in inter-subunit bonds, and upon disassembly of the polymer might become the immunodominant determinants in the isolated monomers.

It has been suggested that in the evolution of viruses the most type-specific antigens became located on the outermost parts of the viral structure [170]. Following this concept, neotopes and metatopes should have developed into highly type-specific antigenic determinants, while cryptotopes should be common for a certain group of viruses. In other words, neotopes and metatopes should be part of a variable amino sequence, and cryptotopes, part of a relatively invariable amino sequence within the evolving amino sequences of the constituent polypeptide chains of viral proteins.

The considerations mentioned above would apply also for components of viral structures located inside virus particles (inner capsids, nucleoprotein cores). Antigenic sites occurring on such structures are expected to be the least specific [169, 170]. For this reason and because of their location, it is believed that these antigenic sites are not involved in eliciting VN antibodies.

Our considerations are valid also for viruses containing lipids and/or saccharides in addition to nucleic acid and proteins. The association of viral protein components with lipids (mediated predominantly by hydrophobic bonds) might result in the appearance of neotopes because of possible allosteric transitions in the lipid-bound proteins. Extraction of the lipids from such viruses would result in disaggregation of the viral envelope and possibly in unmasking the cryptotopes. The hydrophobic sites of the viral proteins formerly involved in the lipid-protein bond may have a tendency to form new inter-protein bonds, leading to aggregates in which the hydrophilic sites are exposed to the surrounding medium and in which the cryptotopes may become hidden again. The exposed antigenic sites on such artificial aggregates may be similar or

identical to the antigenic sites arranged on the surface of the lipid-containing virus.

Saccharides, if present in the viral envelope, may become covalently bound to viral proteins (= glycoproteins) and may function as antigenic determinants, to which the above mentioned theoretical considerations would also apply.

It also must be stressed that the immunogenicity of a protein should be considered in terms of the differences between its antigenic determinants and the antigenic determinants of autologous macromolecules of the species which is being immunized [57]. Previous immunological experience also influences the specificity of the immune response [94, 95, 341]. Furthermore, antibodies against a single antigenic determinant are heterogeneous; they react with different portions of the determinant and differ in their avidity toward the antigenic site. This heterogeneity changes with time following immunization, differs for individuals of the same species and is under genetic control [20, 59]. The combination of antigens with antibodies formed in the immunized subject may lead to the appearance of neotopes and thus to the elicitation of antibodies specific for the particular antigen-antibody (primary) complex [121, 123].

The discussion presented so far was concerned with changes in antigenic determinants resulting from changes in the degree of association of monomers into polymers. There is, however, evidence that such association can lead to changes of antigenicity independent of qualitative changes in antigenic sites. An increase in the size of an antigen can considerably increase its antigenicity [24, 76, 88, 112, 121] and may prolong the persistence of antibodies formed in immunized subjects [329]. This may be explained partly by the greater resistance of aggregated proteins to degradation by lysosomal enzymes [278].

The biosynthesis of antibodies is controlled by the specific antigenic determinants that define the antibody combining sites, and also by the parts of the antigen molecule that define other areas of the antibody structure. Thus it was found that the net electrical charge of antigens profoundly influences the charge of synthesized antibodies [294]. Modifying the charge of antibodies with chemical reagents may considerably affect their virus-neutralizing activity [176]. These charge effects should be borne in mind before chemical reagents, such as formaldehyde or β-propiolactone, which increase the net negative charge of proteins [323], are used to inactivate the infectivity of viruses in order to prepare 'killed' vaccines.

The ideas outlined here may comfort those who have not succeeded in producing 'subunit' vaccines effective against a given viral disease, since they

offer an explanation for the failure and, perhaps, also a way to achieve future success.

### *B. The Ternary System: Virus-Antibody-Cell*

Since we are concerned with the question of effectiveness of viral structural components in inducing in immunized subjects antibodies capable of preventing a viral disease, the mechanism whereby such prevention is achieved should be briefly discussed. To simplify this task, the model *in vitro* system – virus-antibody-cell – should be mentioned first.

The binding of antibodies to the surface structure of viruses does not affect the viral genome. In fact, infectious viral nucleic acid can be isolated from virus-antibody complexes in quantities identical to those from an equivalent amount of intact virus [260]. The binding of antibodies to the surface structure of the virus, however, may prevent the viral genome from reaching in intact form intracellular sites at which the replication of the genome would be initiated. This hindrance may occur at one of these stages: adsorption of the viruses to the cells, penetration (engulfment) into the cells or intracellular uncoating. This subject has been discussed in great detail in a recent review [319], and it remains only to stress the following points:

1. Different types of cells deal with the same type of virus-antibody complex in different ways.
2. The same cell population may react differently to the same virus-antibody complexes under varying physiological conditions.
3. The properties of the reacting antibody (type, charge, avidity) are determinant for the way in which cells will react to the antigen-antibody complexes and score them as 'infectious' or 'noninfectious'.
4. In the case of viruses whose capsid (envelope) is composed of nonidentical structural components, antibodies to the distinct components will have a different effect on the way the corresponding virus-antibody complexes will be handled by cells.
5. Viruses belonging to the same group but to different serotypes may be dealt with by the cells in a distinct manner [53]. This also might apply to the corresponding antigen-antibody complexes.
6. Components of complement present in or added to an antiserum may affect the inactivation of virus infectivity by antibodies [71].

The problems concerning the reaction between viruses and antibodies *in vivo* are even more complex and are beyond the scope of this review. We shall limit ourselves only to the self-evident proclamation that in order to be effective in preventing infection, VN antibodies in sufficient amount must have a

chance to meet the invading virus. Therefore, in certain localized infections, humoral antibodies may be incapable of affecting the course of disease. This task can be achieved only by antibodies reaching the site of ongoing infection. This fact must not be overlooked when vaccines consisting of intact viruses versus those consisting of viral structural components are compared. Vaccination may also result in sensitization to subsequent infection in some cases [52]. In other instances, suppression of the immunologic response may reduce the severity of virus-elicited disease [108]. Therefore, only the response to experimental or natural challenge with infectious virus can be a measure of meaningful immunity.

# III. Enlightening Illustrations

Various aspects of the comparative immune response to complex macromolecules and their constituent components have been studied by different investigators. It is the purpose of the following paragraphs to arrange the results of these studies so that they will complement each other and fit into the framework of ideas outlined in the preceding part of this essay. Such an arrangement, hopefully, may become a guideline for the design of studies on the immunological properties of viral structural components. It seems appropriate to summarize first the results of the elegant quantitative studies on the immunogenic properties of intact flagella and monomeric flagellin from *Salmonella adelaide* [236, 237].

## *A. Intact Flagella and Monomeric Flagellin*

The regulatory role of antigen dose of intact flagella or monomeric flagellin on the development and physicochemical properties of antibodies produced following immunization of rats was studied. Quantitative studies concerning changes in avidity of antibodies were not performed. These studies revealed the following with respect to differences in immunization by flagella and flagellin:

1. While flagella caused an early synthesis of 19 S antibodies and later prolonged synthesis of 7 S antibodies, flagellin failed to induce the formation of 19 S antibodies and the primary antibody response was delayed at all dosage levels used.

2. The peak antibody titers obtained with optimal quantities of flagellin were lower (about 10 x) than those obtained with flagella.

3. No dose-response could be established for flagellin ($10^{-3}$ to 10 μg). The antibody response of individual animals immunized with flagellin varied considerably. With flagella, a dose-response relationship in the range of $10^{-5}$ to $10^{-1}$ μg was found for total antibody formed.

4. Secondary responses were evoked by both antigens only when the second dose equaled or exceeded the first. The dose response curve for the secondary response in animals given the same dose of flagellin as primary and secondary stimulus had a lower slope than a similar curve obtained when flagella was used for both immunizations.

5. Sub-immunogenic doses of flagellin induced immunological memory, which at low dose ranges was equal to or greater than that following similar primary doses of flagella.

These conclusions offer an excellent background for the understanding of results obtained by immunizing experimental animals with preparations of viral structural components *(vide infra)*. Unfortunately, the instructive value of the model is not complete, since experiments using flagella for primary and flagellin for secondary immunization were not performed. This combination might bear some relationship to cases in which subjects who had experienced a previous contact with intact virus were immunized with structural components of the same or a serologically related virus.

### *B. Bacteriophages Qβ and ΦX174*

While the described comparison of immunogenic properties of flagella and flagellin was based on the study of serological responses to these antigens in immunized animals, a different experimental approach was used to assess the antigenicity of Qβ phage, its structural components and reconstituted particles. These antigens were compared by antiserum blocking tests using neutralization of infectious phage (plaque formation) as the indicator system [145]. An antiserum against intact phage was used. The heterogeneity of antibodies (7S and 19S) in the serum and the possible effect of complement on the results were not considered. The comparative relative rates of serum blocking during phage neutralization for intact phage, coat protein monomers, aggregates of coat protein and reconstituted phage particles (reconstituted from coat protein and phage RNA) were 1, $6 \times 10^{-6}$, $2 \times 10^{-5}$ and 0.12, respectively. Although these results suggest the essential role of the quaternary structure for the antigenicity of the phage (appearance of neotopes), it cannot be ruled out that the minor protein component required for the infectivity of reconstituted phage [144] or its proper association with coat protein monomers is necessary for the complete recovery of antigenicity. The fact that the rate of antibody-blocking by the reconstituted particles is about 8 × less than that of authentic phage supports this view. Another possibility which should be considered is that the conformation of the capsid protein monomers may change in the process of their preparation from intact phage, which involves a phenol-extraction step, and the change might affect the antigenic determinant(s) of the monomer. Conformational changes occurred during the reversible depolymerization of the protein coat from bacteriophage *fr* by acetic acid [289].

Detailed studies on the synthesis of 19S and 7S antibodies resulting from primary and secondary immunization of guinea pigs by various quantities of bacteriophage ΦX174 have been reported [334, 335]. Quantitative changes in the avidity of these antibodies in the course of immunization also have been described [97]. These investigations offered an excellent background for similar studies on the subunits of this bacteriophage, which, unfortunately, were never performed. It was shown, however, that the 70S and 6S components found in bacteriophage ΦX174-infected *E. coli*, representing DNA-deficient capsids and capsomers, respectively, were precipitated by an antiserum against intact phage [118]. This indicates the presence of metatopes on the phage surface. It is possible also that cryptotopes exist which are unmasked by thermal inactivation of the phage [269]. It would be of great interest to define the immunological properties of the spikes of the phage [45] and their possible role in eliciting VN antibodies

### *C. Plant Viruses*

The possibility of characterizing viruses immunologically and immunochemically is limited by the amount of knowledge concerning their detailed physical and chemical properties. More information has been accumulated on the tobacco mosaic virus (TMV) in this regard than for any other virus. Consequently, it also became possible to elucidate, at least partly, the chemical nature of the antigenic determinants on the intact virus and the TMV protein subunit. This immunochemical conquest is unique with respect to viruses, and, perhaps, may have escaped the notice of those who are interested in viral structural components as tools of prophylactic immunization.

The capsid of TMV consists of 2,130 identical subunits having a molecular weight of 17,530 each. The subunit is a single polypeptide chain consisting of 158 amino acids whose sequence is known [99, 151]. Detailed comparative studies on the serological properties of the virus and the protein monomers were undertaken. When rabbits were immunized with monomers, the amount of precipitating antibodies directed against the virus was 2 to 8 times less than that when rabbits were immunized with the intact TMV. Both measurements were made when antibody titers had reached peak levels. Anti-TMV antibodies were synthesized for a much shorter period (1 month compared with more than 1 year) when monomers were used. Intact TMV persisted in lymph node cells of the immunized animals much longer than did TMV protein [191]. Unfortunately, no attempts were made to determine the antigen-dose de-

pendence of the antibody response. With lower amounts of TMV protein (10 µg instead of 5 injections of 20 mg each), apparently no precipitating antibodies against TMV were formed [199], although equal amounts of intact TMV induced antibody synthesis. Interestingly enough, RNA-free protein rods (capsids) artificially reconstituted from the monomers seemed to be as equally inefficient as the monomers in inducing anti-TMV precipitating antibodies. In contrast, TMV reconstituted artificially from TMV RNA and protein monomers seemed to be as immunogenic as native TMV. The role of RNA in the immunogenicity of TMV rods remains unclear.

The assembly of TMV protein monomers into either RNA-free polymerized TMV protein rods (capsids) or into reconstituted TMV apparently caused the appearance of neotopes. This was borne out by the following: anti-TMV serum adsorbed with protein monomers still reacted with TMV, artificially reconstituted TMV and TMV protein capsids in antigen-antibody precipitation tests. On the other hand, antiserum prepared against the monomers, when adsorbed with TMV, reconstituted TMV or TMV capsids, ceased to react with monomers [322]. The indication of differences in antibody binding sites on intact TMV and on TMV protein monomers was further supported by results of experiments designed to identify the antigenic determinants and establish their location in the amino acid sequence of the TMV protein. Different peptides prepared by proteolytic digestion of TMV protein were purified and tested for their inhibitory activity on the precipitation of TMV by antiserum against TMV. Of 20 peptides covering the whole amino acid sequence of the polypeptide chain, only 4 had an inhibitory effect. These 4 peptides represented about 15 to 20% of the total amino acid sequence of TMV protein [6]. It was found that 3 peptides corresponding to the C-terminal amino sequence of TMV (positions 142 to 158, 151 to 158 and 153 to 158) had an inhibitory effect; this agrees with the observation that the C-terminal amino acid (threonine) is exposed on the surface of intact TMV. An antiserum against the C-terminal hexapeptide Thr-Ser-Gly-Pro-Ala-Thr, coupled to bovine serum albumin as carrier, precipitated TMV and inactivated its infectivity [7]. Antisera against the C-terminal penta-, tetra-, tri- and dipeptides, and against threonine had similar properties but showed a slightly decreasing tendency to neutralize TMV as the size of the haptenic group decreased [8, 9].

Another peptide (positions 93 to 112), designated as peptide 8, did not inhibit the precipitation and complement fixation reactions of TMV and TMV antiserum, but inhibited the fixation of complement by TMV protein (monomer) with TMV protein antiserum [21]. The binding of this peptide to antibodies against TMV protein was confirmed using a [$^{14}$C]-labeled peptide [22].

This peptide could be further degraded and smaller peptides, one of which was a pentapeptide, (positions 108 to 112: Leu-Asp-Ala-Thr-Arg) exhibited specific immunological binding with antibodies to TMV protein [370, 371]. Furthermore, a polypeptide corresponding to the C-terminal decapeptide of peptide 8 was synthesized and had specific immunological activity [314]. When these peptides were used to immunize guinea pigs, no homologous antibodies or antibodies precipitating TMV protein were synthesized [310]. Perhaps this failure might have been prevented if the peptides had been coupled to a protein carrier. The amino acid sequence of peptide 8 is probably not available at the surface of intact TMV and becomes exposed only upon disaggregation of the nucleocapsid and the release of monomers [99]. Undoubtedly, the overall immunological specificity of TMV protein and TMV is defined by the spatial folding of the polypeptide chain in the TMV protein subunits, whether isolated or in the aggregated state. The isolation of TMV (TMV protein)-specific haptens, described here, was a partial success which, hopefully, will be followed by stereochemical delineation of antigenic determinants.

The appearance of neotopes resulting from the assembly of capsomers into viral capsids or into smaller aggregates, and the unmasking of cryptotopes upon degradation of the viral capsid into monomers, was also demonstrated for bromegrass mosaic virus [342] and suggested for barley-stripe mosaic virus [15].[1]

The role of viral RNA for immunogenicity was demonstrated not only for TMV but also for turnip-yellow mosaic virus (TYMV) [199]. The empty capsid of TYMV was less immunogenic in both primary and secondary responses (measured by the titer of precipitating antibodies in sera of immunized rabbits and rats) than either infectious or noninfectious virus containing the full complement of viral RNA.

### *D. Hemoglobin and Myoglobin*

In the previous paragraph it was shown that the known amino acid sequence of TMV protein permitted the characterization of at least some TMV (TMV protein)-specific haptens. It will be interesting to learn how the spatial conformation of a protein as well as the amino acid sequence will determine

[1] An immunologic disparity was also demonstrated for potato virus X and its protein subunits [SHEPARD, J. F. and SHALLA, T. A.: An antigenic analysis of potato virus X and of its degraded protein. I. Evidence for and degree of antigenic disparity. Virology *42:* 825–834 (1970)].

its immunologic properties. We shall now summarize briefly the results of research concerning the antigenic sites on hemoglobin and myoglobin, the amino sequence and detailed conformation of which have been elucidated [35, 271], and suggest the possible implications of these results for the study of viral antigens.

The basic structure of human and other mammalian hemoglobins is the same. They consist of 4 polypeptide chains (2 α-chains consisting of 141 amino acids and 2 β-chains, of 146 amino acids). In human fetal hemoglobin, the β-chains are replaced by γ-chains, which also consist of 146 amino acids, but there are 39 points of difference between the β- and γ-chains [35]. Antibodies against the isolated α- and β-chains from human hemoglobin A fix complement with the homologous antigens and with hemoglobin A. There are no immunologic cross-reactions between the α- and β-chains. Quantitative complement fixation tests revealed no differences between the binding of anti-α antibodies to free α-chains or α-chains assembled into hemoglobin. However, when β-chains of hemoglobin were replaced by β-chains from other species, or by γ-chains, the complement fixation by anti-α antibodies and the corresponding hybrid hemoglobin decreased. The reaction of anti-β antibodies with free β-chains was much stronger than with β-chains assembled with α-chains in native hemoglobin, suggesting that cryptotopes might have been buried in the assembly process. The replacement of the α-chains from other species to form hemoglobin hybrids did not result in a further decrease of complement fixation between anti-β antibodies and assembled β-chains. Anti-β serum absorbed with hemoglobin A was still capable of giving complement fixation with free β-chains, supporting the idea that cryptotopes are present on these chains [263]. Substitution of a single amino acid in mutant chains in either α- or β-chains led to a considerable decrease of complement fixation by homologous antisera. Differences in complement fixation between oxy- and deoxy-hemoglobin were also observed and explained by differences in quaternary structure involving a distinct arrangement of the α- and β-chains in the 2 forms of hemoglobin. Amino acid replacements in the chains also affect the quaternary structure of the molecule [29]. Thus it becomes evident that minor changes in the amino acid sequence of a polypeptide chain may affect not only the specificity of antigenic determinant(s) located on this chain, but also of those on a second chain in which no amino acid replacements occurred if the second chain is associated with the chain having an altered amino sequence. Such changes might account for a greater chance that complex proteins (viruses) consisting of several different polypeptide chains will undergo antigenic variations, thus contributing to the emergence of different serotypes of the same virus.