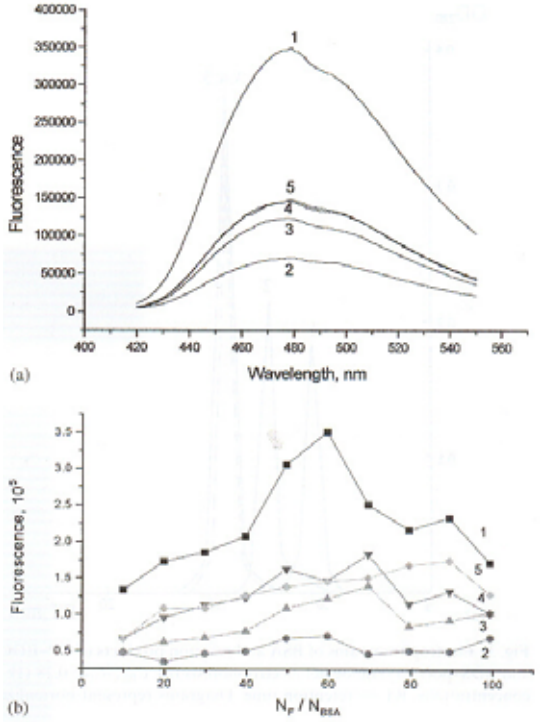



**Figure 111.** (a) Fluorescence spectra of pure BSA (1) and conjugates in the mixture with fluorescamine at different $C_{BSA}/C_{CP}$: 0.28(2); 0.59(3); 1.14 (4); 1.70 (5) $C_{BSA}$ = 0.07 mg/ml; $C_F$= 1.8 x 10 -2 mg/ml; different polymer concentrations. (b) Intensity (1 max) in the second excitation maximum of fluorescamine bound to BSA (1) and reaction products (2-5), preparing at different initial ratios vs. concentration of fluorescamine $C_{BSA}/C_{CP}$: 0.28 (2); 0.59 (3); 1.14 (4); 1.70 (5); CBSA = 0.71 mg/ml; different polymer concentration.

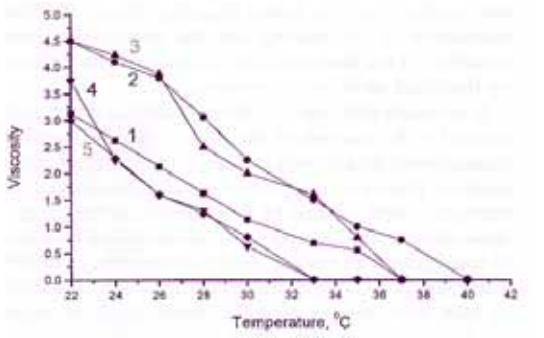



**Figure 112.** Dependence of viscosity (sp/c, dı/ı) of solution of pure CP (1) and conjugates on temperature, preparing at different ratios: 0.28 (2); 0.59 (3); 1.14 (4); 1.70 (5) Ccp = 0.25 ı/dı, different protein concentrations. Phosphate

It was found that the phase state of CP-BSA conjugates at low concentrations was

independent of temperature rise, remaining soluble in water. On the contrary, more concentrated solutions of CP-protein conjugates as free CP solutions retain a cloud point around 26°C in the phosphate buffer (pH 7.2). Therefore, it can be concluded that protein conjugation does not significantly influence the cloud point or the thermally induced precipitation of poly(NIPAAm-co-AA). These findings agree with the results of the study by Chen et al. [156], who found that the conjugation of proteins with poly(NIPAAm) did not change the LCST of the polymer. Besides, Cole et al. [157] did not actually see the precipitation (i.e. the cloud point) of the poly(NIP AAm)-monoclonal antibody conjugate, probably because the concentration they use was too low. What they observed was the collapse of individual polymer chains using laser light scattering in the dilute solution.

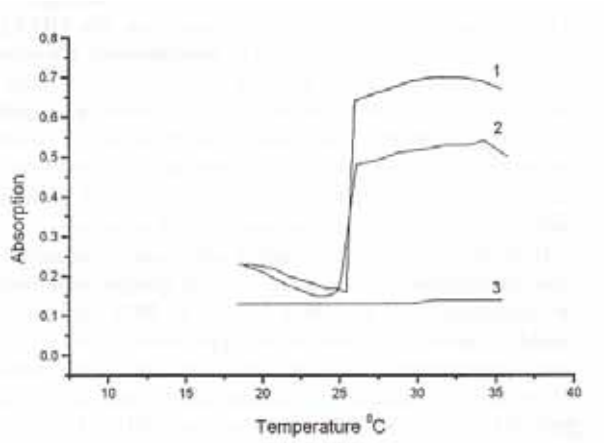
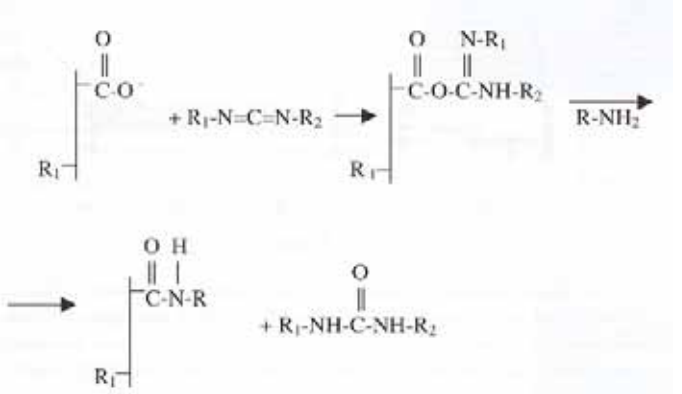


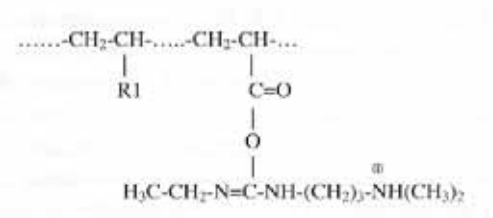

**Figure 113.** Temperature dependence of $OD_{500}$ values for the solutions of CP (1) and CP-BSA conjugates at different concentrations: 1.0 g/dl (2), 0.25 g/dl (3); phosphate buffer (pH 7.2); $C_{BSA}/C_{CP} = 0.28$.

The most important chemical modification reactions of carboxyl groups utilize the carbodiimide-mediated process. In the presence of an amine, carbodiimides promote the formation of an amide bond in two steps. In the initial reaction, the carboxyl group adds to the carbodiimide to form an o-acylisourea intermediate with an amine for the polymeric carboxylic acids, yields the corresponding amide:




Water-soluble carbodiimide- 1 -ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) $CH_3CH_2$-N-C=N-$CH_2CH_rNH$+ ($CH_3h(R_1$-N=C=N-$R_2$) contains two radicals, one of them is

positively charged ($R_2$) and at the activation of anionic polyelectrolytes by these carbodiimide the carboxyl group of polymer molecules transform to positively charged ester derivatives and we practically have to deal with cationic polymers. In our case, the chemical structure of copolymers activated with EDC is likely the following:




where $R_1 = CONHCH(CH_3)_2$

When BSA solutions are added to activated CP (CP-EDC) solutions, one can asstime that first the formation of the electrostatic (and hydrophobic) interpolymer-protein complexes occurs as a most rapidly proceeding process and then the condensation between carboxyl and amino groups of components (covalent conjugation) occurs.

HPLC, electrophoretic, fluorescence and viscosimetric results suggest that the mode of binding of BSA to activated polyions depends upon the weight concentration ratio *(r)* of protein molecules to CP. When *r* is smaller *(r* < 1), the free and bound CP (conjugate with BSA) is coexistent and free BSA in these solutions is not detectible. These results suggest that for $r < 1$ values, the CP molecules are either covalently bound with BSA or free, an indication of a cooperative binding mechanism. A similar nonuniform distribution of BSA among polyelectrolyte (hosts) in electrostatic complexes was observed earlier by Kabanov and Mustafaev [34,35] and lately in [153]. Cooperative binding has also been observed in complexes of oppositely charged synthetic polyelectrolytes [34] and native calf thymus DNA with cationic polypeptides [73]. We show that the values of binding amino groups in composition of bioconjugate molecules (degree of binding, β) depend on the *r* conditions, have a maximum values at $r < 1$, then decreased and remains essentially constant for *r* 1 values. This means that each incremental addition increase in the mass of bioconjugate. Because of the constant degree of binding it can be assumed that at $r >$ 1, the structure of the conjugate is constant and the concentrations of bound BSA will increase proportionately as well. This process is consistent with cooperative binding. Although the present results do not provide any direct information on the structure of polymer-protein conjugates, some speculations concerning conjugate structure can be made. When BSA complexes with oppositely charged activated CP is formed, charge neutralization makes the bound protein more hydrophobic. Consequently, hydrophobic interactions between adjacent bound proteins can be a driving force for the cooperative behavior proposed in this study. However, after condensation reaction within the polycomplex particles, and removal of positively charged carbodiimide derivative from the solution by dialysis the volume and the asymmetry of conjugate particles increase because of occurrence of repulsive interactions the between negative charges of bound protein molecules and carboxylic groups of polyions and bound protein molecules.

Figure 114 shows the schematic representation of the carbodiimide-induced conjugation of BSA with polyanions and hypothetical structures of the CP-BSA conjugates. The character of the binding depends on the [BSA]/ [polymer] ratio and two types of bioconjugate particles are formed: at $r < 1$, the protein molecules in the structure of conjugate particles are densely covered as a shell by polymer chains and practically "fenced off" from water environment; at $r > 1.0$ forming conjugate particles possess more friable structures in which protein molecules are practically exposed to the solution.

It is reasonable to conclude from these observations that the negatively charged carboxylic groups remaining in composition of poly(NIPAAm-AA)-BSA conjugates could eventually prevent phase separation if the conjugate concentration is very low. At the same time,

when the temperature is raised above the LCST of poly(NIPAAm), the precipitating poly(NIPAAm) segments of the conjugates will flocculate together if the concentration is high enough, similar to free poly(NIPAAm-AA). In this study, we see the thermally induced precipitation and inoculation of conjugates in all studied $C_{BSA}/C_{CP}$ ratios since we used more concentrated solutions.

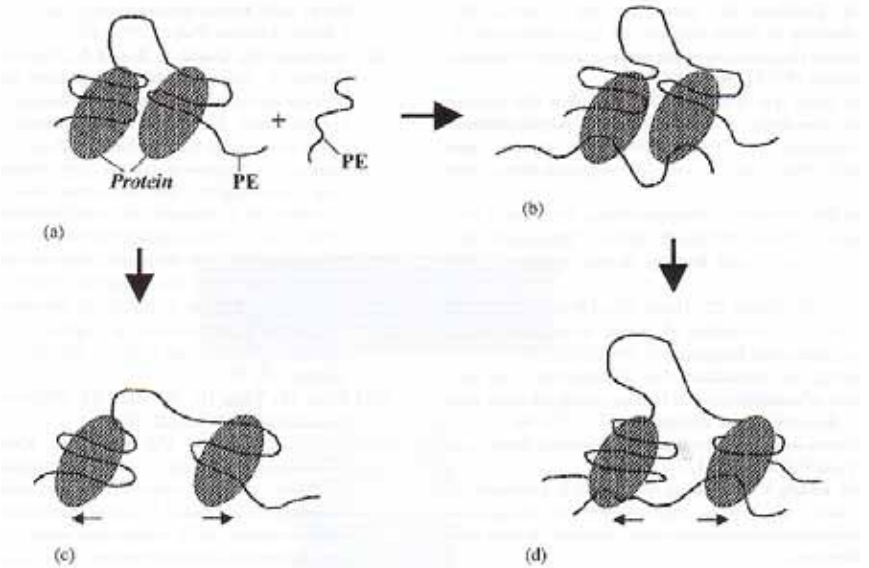



**Figure 114.** Models of possible organization of CP-BSA conjugates and structure of conjugates: polycomplex particles of CP-EDC-BSA *(a,r* > 0.3), *(b,r* < 1); conjugates after condensation reaction *(c,r* < 1), *(d,r* > 1).

A single immunization with these conjugates increases specific immune responses to BSA, whereas, the level of the antibody titers does not depend on the composition of soluble conjugate molecules. In the aqueous solutions, poly(NIPAAm-AA) transforms from hydrophilic to a hydrophobic state at 36.5°C (body temperature). The precipitation of conjugate molecules leads to "leveling off" of the effect of conjugate composition on their immunogenicity and the hydrophobic aggregation site on the surface of poly(NIPAAm-AA)-BSA molecules would increase the absorptive capacity of BSA (antigenic determinants) on the immunocompetent cells. The efficiency of such "forced" interactions of conjugate aggregates (high epitope density) is high enough for the immune response (see below).

## 5. BIOPOLYMER SYSTEMS IN IMMUNOLOGY

The principle of development of vaccines discovered by Pasteur in 1881 still serves as a basis for the whole immunology practice. Pasteur's vaccine is a suspension of attenuated or killed microbes which do not provoke a disease but can cause the protective reaction of the organism, i.e. more or less stable immunity to living pathogens. Special proteins, antibodies, which are one of the products of the immune reaction, bind specifically to antigens, foreign proteins and polysaccharides of the pathogen and thus, block them. The immune response strength is characterized by the number of antibodies or antibody-forming cells (AFC) produced by the organism in response to introduction of the antigen.

For the last 100 years the development of practical immunology, which provided

humanity with powerful weapons against many destructive infections, was mainly based on the development and improvement of the Pasteur method of vaccination. But this method though being of fundamental importance and great productivity has, at least, two restrictions.

The first restriction is as follows. The whole or partly destroyed microbes, which are introduced into the organism, are multi-component complex systems. Alongside with the production of antibodies necessary to destroy the infection, they inevitably inflict the production of multiple varieties of ballast antibodies. The immune system here works idIe to a great extend and this often causes undesirable side effects. The attempts to overcome this drawback by vaccination with previously extracted individual microbe antigens (pure proteins and polysaccharides) are not successful: individual antigens do not ensure strong enough immune protection.

The second restriction is stilI more serious. The strength of the organism protective reaction to this or that antigen is genetically programmed and controlled by special immune response genes (Ir-genes). Such genetic control means that depending on the structure of its genome-, the species can produce high or low response to the introduction of particular antigens or even cannot respond at all. That is, apparently, why it is so difficult to prepare effective vaccines against some dangerous infections by way of inactivation of corresponding viruses or bacteria.

Thus, to overcome these restrictions, one must find the way of the enhancement of immunogenicity of individual antigens (or their active fragments - haptens) independent from genotype of the organism, i.e. avoiding the genetic control. Then, low responder species would be converted into high-responder ones. In recent years certain achievements in solving this problem were made by a joint effort of a group of immunologists and immunogeneticists headed by R.V.Petrov and R. M. Khaitov and a group of polymer chemists represented by V.A. Kabanov and M.I. Mustafaev [158-186]. Cells of two types - B-lymphocytes and T-lymphocytes are known to be the most important components of an animal and human immune system. The former are formed from the stem cells in the mammal bone marrow. The final maturation of B-cells occurs both in bone marrow and in the spleen. The latter are formed in the thymus and also originate from the stem cells of bone marrow. The surface protein receptors of B- and T-lymphocytes "recognize" antigens introduced into the organism. B-lymphocytes are smaIl antibody producing factories. Its own B-lymphocyte clone corresponds to each antibody variety. The set of B-lymphocytes, which can be formed in the organism, is wide enough to react to all natural antigens and produce corresponding antibodies against them. But in majority of cases the signal of T-lymphocytes helpers is also necessary to initiate the "production". In order to send such mitogenic signal, T-lymphocyte should also "recognize" the antigen. But unlike B-lymphocytes, T-lymphocytes set of each species is individual, limited and genetically determined. That is to say, the genetic control over the immune reaction strength is realized by means of T-lymphocytes. Thus, to avoid this, one should first find the way of activating B-lymphocytes without T-helpers participation.

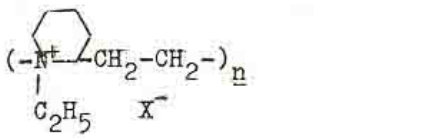
### 5.1. Polyelectrolyte Immunomodulators

Pilot studies of the adjuvant activity of non-natural (synthetic) polyelectrolytes demonstrated that some high molecular weight compounds of polyelectrolyte origin, e.g., macromolecules bearing positive and negative charges or capable of being charged at physiological values of the ionic strength and pH (polybases, polyacids and their copolymers), despite the differences in their electric charge or chemical structure (or even in the chemical nature of their constituent components) displayed a similar immunostimulating activity. At the same time neutral and uncharged polymers as well as polymers incapable of acquiring an electric charge in aqueous media (for example, poly-N-vinylpyrrolidone, polyethylene glycol, dextran, etc.) had no effect on the immune response to classical antigen-sheep red blood cells (SRBC) and thus exhibited no

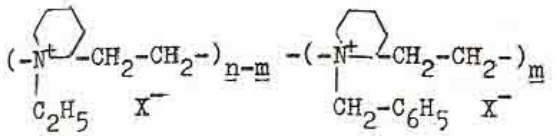
adjuvant activity. Monomeric derivatives of polymeric adjuvant components were also devoid of immunostimulating activity. Quite an opposite effect was observed when copolymers of acrylic acid with methylvinylpyridine whose polymeric chains contain both acidic and basic groups produced immunosuppressor effects and inhibited the immune response by inducing selective elimination of T-B interactions. This finding led the authors to suppose that the action mechanism of polyelectrolyte adjuvants is not directly linked with the fine peculiarities of their chemical structure (nonspecificity towards the monomeric link of PE) and that their immunomodulating activity is due to some common properties that are conferred on them by their polymeric origin.

**Polyelectrolyte Immunostimulants.** The activation of the immune system is known to be achieved with the help of great variety of different polyelectrolytes (PE) both of polycationic and polyanionic nature [9,9a,187-195]. Synthetic PE structural analogues of which do not exist in nature are of particular interest because they are not immunogens, i.e. they do not cause the production of unnecessary antibodies against themselves.

The list of synthetic PE immunostimulants (adjuvants) used by Kabanov and Mustafaev includes polyacrylic acid (PAA), copolymers of acrylic acid with vinylpyrrolidone of various compositions, poly-4-vinylpyridine (PVP) and its quaternary salt s (PVP-R), poly-2-methyl-5-vinylpyridine (PMVP), quaternary polyconidine salts (PC-R), in particular:



(PC-$C_2H_5$)



(PC-$C_2H_5$, -$CH_2$-$C_6H_5$)

m/n = 0.2

Despite of considerable differences in structure and even in the very chemical nature of the repeating monomer units, PE adjuvant activity does not differ much if their chains are long enough. They all increase the number of AFC by several times (usually not more than by a factor of 10). PE monomeric analogues (propionic acid, ethylpyridine etc.) are not active.

The establishment of ability of different PE immunostimulants to substitute, at least partly, T-helpers function [196,197] represented the important stage in solving the problem discussed above. So-called B-mice, i.e. the mice that do not have T-cells, T-helpers in particular, were used in these experiments. The immune response strength was determined by the number of AFC produced in mouse spleen in response to the joint introduction of a standard dose of sheep erythrocytes (SE) as antigen and PE. As it follows from Table I, B-mice cannot practically develop the immune response to SE, what can be accounted for by T-helpers deficiency. However, the introduction of the antigen together with PE causes a considerable immune response.

Thus, the presence of PE compensates for the deficiency of T-helpers, i.e. the immune response becomes T-independent (here and further terms "T-dependence" or "T-independence" mean only the presence or absence of a considerable immune response in the absence of T-lymphocytes). It is important that PE, when introduced into the organism, induce, at the same time, the transformation of many various B-lymphocytes clones into AFC (specific in relation to SE, donkey and horse erythrocytes, trinitrophenol on serum albumin etc). In other words, PEs are nonspecific polyclonal activators of B-lymphocytes [198,199].

The data presented above as well as some additional immunological data on which we cannot dwell here (for details, see review [228] lead to the conclusion that PE can affect the immune system avoiding T-helpers.

**Table 10.** Influence of Polyelectrolytes on B-mice antibody Genesis (SE dose $I0^7$ per mouse, polymer dose 50 mg/kg

| Polymer | M.W. | Number of mice | Number of antibodies in the spleen |
|---|---|---|---|
| - | - | I2 | 30 ± I0 |
| PVP | 50000 | I5 | 500 ± 80 |
| PAA | 80000 | I3 | 800 ± I50 |
| C-AA•VPD (55:45) | I00000 | 9 | 400 ± 500 |

Even in the early-published papers [191] we assumed the determining role of cooperative PE adsorption on outside membranes of immune cells in immunogenesis stimulation. This assumption is in agreement with recently published data on dependency of immunostimulation activity of different PE upon their degree of polymerization (n) [172]. As it follows from Figure 115 the adjuvant effect appears only when “n” exceeds some "critical" value", sharply increases and, then, practically reaches the limit. The similar dependencies were observed for polymerhomologous series of PAA, PVP and PVD-$C_2H_5$. Sensitivity of the effect to the structure of PE monomer unit manifests in "critical" value of “n” characteristic for each PE.

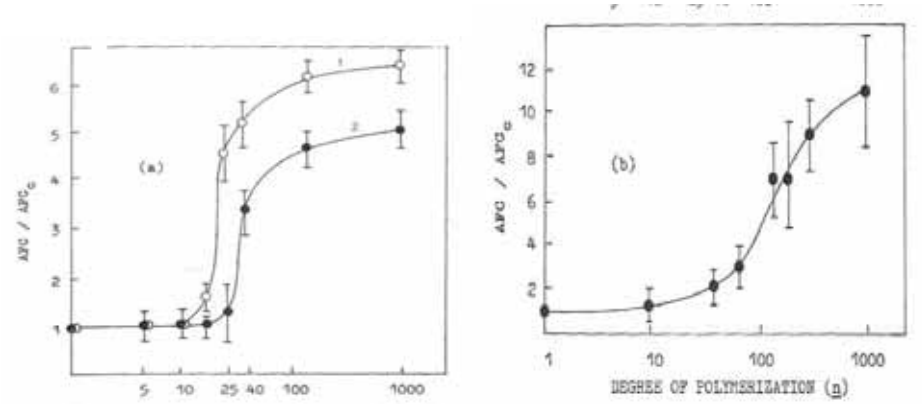



**Figure 115.** Dependencies of the relative number of antibody-forming cells (AFC) in the B-mouse spleen on the degree of polymerization of quaternary polyconidine salts (a) and PAA (b): 1- (PC-$C_2H_5$); 2- (PC-$C_2H_5$, -$CH_2C_6H_5$). The dose of the PE immunostimulants is 50 mg/kg mice were immunized by SE in dose of $5.10^6$. $AFC_C$ is the number of AFC in the blank experiments

At the same time, ”n” was found to depend on the conformation peculiarity of PE, and it decreased during the transition from flexible-chain PE (PAA,PVP,PVI) to rigid-chain ones (PC). Macromolecules of PC and its derivatives were less labile due to the presence in the main chain of their macromolecules of cyclic fragments, which start to “operate” at much lower degrees of the macromolecule polymerization.

A detailed investigation of structure and immunostimulating properties of some water-soluble complexes of different PE with low-molecular weight “additives” (hydrophobic lateral

radicals, transient metal ions and surface active substances-sodium dodecyl sulfate (SDS) was carrying out by Mustafaev [9,9a,26,39,85].

Figure 116 shows the immunomodulating properties of PVP(Ro,$R_n$) polycations relative to the number of lateral cetyl radicals, β, and to the length of side alkyl radicals, correspondingly (see section 1).

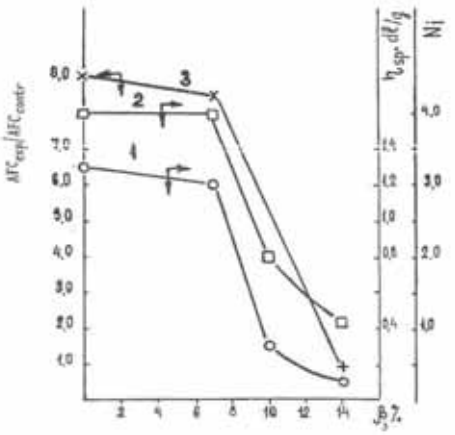



**Figure 116.** The dependence of the relative number of AFC in the spleen cells of SRBC-immunized mice on the number of lateral cetyl radicals ($\beta$) in the PVP($R_0$,$R_{16}$) molecule (3). 1 - dependence of viscosities (hydrodynamic sizes) of PVP($R_0$,$R_{16}$), on $\beta$ 2 - dependence of the number of protein molecules ($N_i$) bound to one PE molecule, on $\beta$ .

PVP(Ro,$R_8$) and PVP(Ro,$R_6$) injected to SE-pre-immunized mice stimulated the AFC production in practically the same degree as PVP. However, PVP(Ro,$R_{10}$) had a far more pronounced stimulating effect on antibody genesis than PVP(Ro,Rn) with $n \leq 8$.With a transition from $R_{10}$ to $R_{16}$ the degree of intramolecular compactization of PVP increased and ,as can be seen from the results depicted in Figure 116,the AFC level in PVP(Ro,$R_{16}$) injected animals was decreased in comparison with PVP(Ro,$R_{10}$) injected mice. It can be seen from the data in Figure 116 that the adjuvant properties of the polycation chains depend on the β and are correlated with the conformational transition from the coil to the compact structure in the polyelectrolyte molecule. The analogous behaviors were obtained also for the PE-Me and PE-SDS systems [9,183-186]. Data from physico-chemical analysis of the PE behavior in aqueous solutions containing low-molecular weight complex-forming "additives" as well as immunological data provide compelling evidence that in all systems under study the mechanisms underlying the immunomodulating effect of polyelectrolyte complex are universal, being coupled with the structural and chemical transitions and conformational state of PEC.

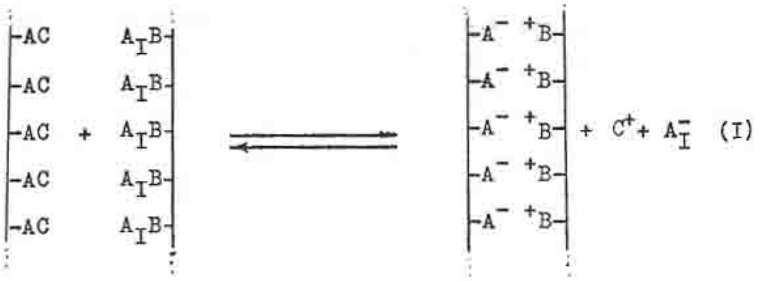
It is known that the constant of cooperative binding of macromolecules is known to be an exponential function of "n" and in the first approximation can be expressed as follows:

$$K=\exp(-\Delta G_m .n/(RT))$$

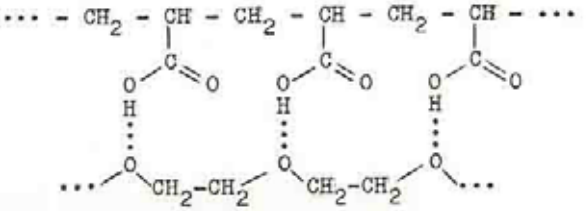
Where ΔG - Gibbs free energy per one unit of the chain. Correspondingly, the degree of binding as a function of n sharply increases from a value, which is close to 0 up to 1 in a rather narrow range of "n". So, the phenomenon appears to resemble a critical one. Therefore, the establishment of the critical character of the dependence of the immunostimulating effect of PE on the chain length has a decisive significance in evaluation of reliability of the above expressed hypothesis.

Physico-chemical foundations of this hypothesis spring from the studies of cooperative reactions between complementary charging PE and PE with proteins, which result in formation of

interpolyelectrolyte and interpolyelectrolyte-protein complexes. Oppositely charged polyions and proteins in such complexes bind with each other by means of multisite sequence of salt and hydrogen bonds, hydrophobic interaction and etc. [200,201]. Interpolyelectrolyte reaction can be represented in the scheme:

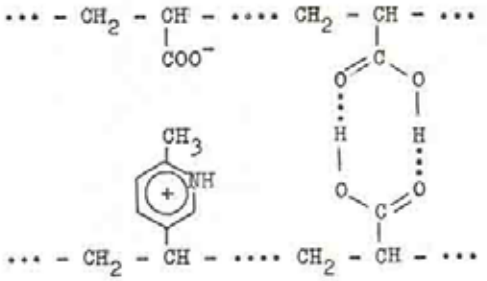



where: -AC is polyacid (or protein ) or its salt unit, $-BA_I$ is polybase (or protein ) or its salt unit, C is cation (in particular case - proton), $A_I$ is anion (in particular case - hydroxyl ion). In some polymer complexes the multisite interaction of components is realized due to formation of hydrogen bonds, for example, in PAA (or polymethacrylic acid)-polyethylene oxide complex:



PAA (or polymethacrylic acid)-PVPD complexes, etc. (see [202]).

Likewise, interpolymeric complexes are also known in which the multisite system of interacting units includes both salt and hydrogen bonds such as, for example, a complex of PAA with acrylic acid and 2-methyl-5-vinylpyridine copolymer [203]:



In the given case, the macromolecule of the copolymer in the polycomplex plays a role of a heterofunctional cooperative partner with respect to PAA homopolymer.

In recent years there was a detailed investigation of structure and properties of some water-soluble complexes of synthetic linear PEs both of cationic and anionic nature with globular proteins and, in particular, with blood serum proteins (albumin, gamma globulin (see section of "Polyelectrolyte-Protein Complexes" in this review) [33-39,60-66,204-207]. Distinctly expressed cooperative character of protein binding with polyions was shown. A role of a cooperative partner, with respect to a linear polymer is played by the totality of functional group*s* at the surface of a protein globule. In terms of multisite cooperative interaction, the surface of a protein globule is a heterofunctional sorbent. Bovine serum albumin (BSA), for example, in the neutral

pH region, forms complexes with polycations, also with some polyanions and with some amphoteric linear polyelectrolytes. Complexing of BSA with PVP($C_2H_5$) at pH=7 is the reaction of type I. It goes with formation of salt bonds.

However, depending on the nature and chemical composition of the chain of the linear partner, in conjunction with the salt bonds essential contribution in stabilization of complexes can further be brought about by hydrogen bonds and by hydrophobic interaction. Complexing of proteins with linear PEs, in fact, consists of a "gluing" of more or less extended molecular chains to the surface of protein globules. Depending on the degree of polymerization in otherwise equal conditions, one molecule can “glue” itself to one or to several globules. In the last case, a complex particle represents an agglomerate of protein globules entangled in linear polyion. In this case, protein molecules can link up with each other. It is important to stress, that a linear PE chain forming a complex with protein molecules does not bind with them in all of its units. A complex particle stilI contains sufficiently long fragments of a linear chain preserved in the form of loops or free ends, which secure the potential possibility of additional multisite binding with other complementary species.

A multisite adsorption of PEs at the surface of colloidal particles is also related to a category of cooperative phenomena, which in their general features are analogous to the formation of interpolymeric complexes [172]. It is known, in particular, that the polycations strongly adsorb on the negatively charged surfaces of colloidal particles. The same applies to the polyanions in their relation to the positively charged colloids.

The dimensions of the cells of the immune system are in the order of 10 μm, that is to say, they are in the dimensional region of particles of typical organic and inorganic sols. The portions of polymeric chains can be adsorbed on the outcoming membrane proteins as well as on polysaccharides fragments of glycolipids and glycoproteins, which represent a considerable part of the overall membrane surface. The polar "heads" of lipids, which form an outer surfaces of a double layer, contain anionic and cationic groups as well as the groups which are capable of hydrogen bond formation, and which can also serve as the centers of adsorption. Certain phenomena of agglutination (sticking) of erythrocytes [208,209] and thrombocytes [210-213] by polybases in terms of their physicochemical aspect are fully analogous to flocculation of ordinary "nonliving" sols by PEs

Thus, the surface of cells formed by outer membranes, is, generally speaking, a universal multisite heterofunctional sorbent for PEs.

The assumption of the nonspecific activation of B-lymphocytes by adsorbed linear PEs agrees with the obtained experimental data. In the *in vivo* experiments, it was shown, that, PAA or PVP introduced in a spleen cell culture of intact mice, increase by 2,5-3,5 times the velocity of the cell division (with respect to the velocity of incorporation of H–thymidine in the DNA composition [214]. It was found, in particular, that PAA selectively activates fraction of the mouse spleen cells, strongly enriched with B-lyphocytes and does not show a mitogenic effect on T -cells. The activation of DNA synthesis in B-lymphocytes begins within 24 hours after the introduction of PAA polyanions [215]. Polycations, on the contrary, are characterized by greater universality of mitogenic effect (i.e. by less selectivity).

What does the mechanism of activation of the immune system cells by ionogenic macromolecules consist of?

Macromolecules of PE entering the blood within the composition of multicomponent biological mixtures interact, primarily, with blood plasma proteins, polysaccharides and nucleic acids as well as with the surface of blood sells; therefore their immunological activity may be manifested in different ways.

**One of putative mechanisms** of the adjuvant effect of linear PE is the adhesion of immunocompetent cells via the multipoint adsorbtion of linear macromolecules on the cell membrane surface. Evidence in favour of this hypothesis can be derived from the fact that cooperative reactions of the macromolecular substitution type facilitate the interactions of every

macromolecule circulating in the blood and coming into contact with many other molecules before it reaches a certain local thermodynamic equilibrium.

Kabanov assumed [216] that the segments of the chains adsorbed on the cell membrane can considerably change its properties such as permeability for ions and other low-molecular substances, characteristics of lipid matrix fluidity and even induce a phase transition in lipid layer. Any of such change may serve as a source for mitogenic signal.

What is the mechanism of ionic permeability formation? Generally speaking, the potential centers of cooperative sorption of ionogenic macromolecules can exist either on the lipid bilayer surface or on outcoming membrane proteins. In fact, model lipid membrane, which does not contain proteins (lecithin black films) adsorbes polyions. An indication of that is the change of membrane electric potential when the PE solution is added [216]. However, their adsorption in this case is not followed by appearing of ionic permeability of the bilayer. Therefore membrane proteins distributed in the lipid matrix are most likely to participate in formation of nonspecific ionic channels when interacting with polymer. It was suggest, that the adsorption of the polyions on real membrane results in formation of protein globules clusters like it happens in case of complexing in solution [34]. Electronic microphotograph of longitudinal sections of the membrane previously treated with polycations confirm this hypothesis (Figure 117). The process of protein cluster self-assembling is schematically presented in Figure 118. The channels responsible for ionic permeability are very likely to appear on the boundaries of joint protein globules.

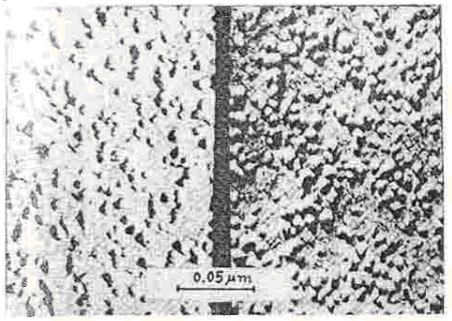



**Figure 117.** Electron micrographs of longitudinal section of the lymphocyte membrane before (left side) and after (right side) PAA solution treatment

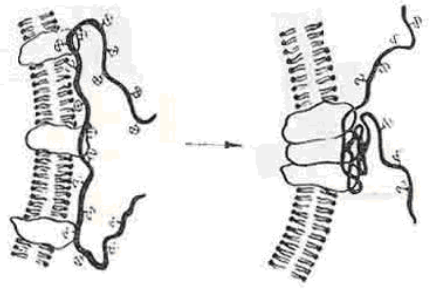


**Figure 118.** Hypothetical scheme of PE-membrane proteins interaction

How does the lymphocyte react to the presence of nonspecific PE - ionophore on its surface? Taking the obtained data into consideration authors suggest that PE adsorbed on plasmatic membrane acts as a trigger in Iaunching the mechanism of cell division. The adsorption goes with formation of nonspecific transmembrane ionic channels and emergence of corresponding transmembrane ionic flux. The disturbance of a cell homeostasis leads, in its turn, to switching on of enzyme ionic pumps (ATPases) to compensate for the $K^+$ deficiency and $Na^+$ and $Ca^+$ excess inside the cell. These are very likely the first stages of succession of molecular events, which, in the long run, lead to the accomplishment of the whole mitotic cycle. It is significant that the dependencies of ionophore and immunostimulant activity of PE on its degree of polymerization perfectly correlate with each other. Macromolecules obtain the capability of induction of transmembrane ionic flux and activating of immune cells only if their length exceeds a certain "critical" value (Figure.119, compare with Figure 115).

The described above mechanism of cell activation as applied to B-lymphocytes, can probably explain the ability of PE adjuvants to substitute the T-helpers function in the immune

response to typical T-dependent antigens. B-lymphocyte receives a necessary nonspecific mitogenic signal from polyion and thus, does not need a mediator, which is usually produced by a corresponding T-helper only after it "recognizes" the antigen.

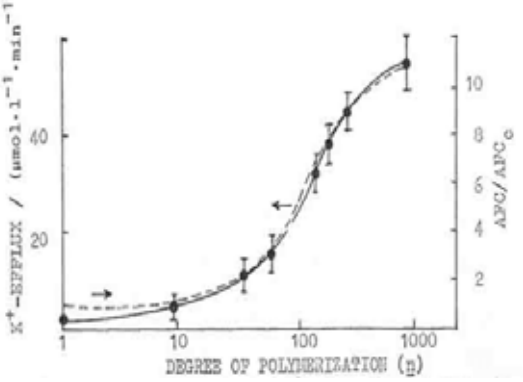



**Figure 119.** Dependencies of $K^+$ efflux through B-lymphocyte membranes in vitro and relative strength of the immune response in vivo on PAA degree of polymerization. Immunization of mice by SE

We proceeded from that an essential role in the manifestation of immunological activity of PE belongs to a proteinoceous factor, since the PE molecules entering the blood predominantly interact with plasma proteins that are present there in large excess. The PE-protein complexes formed thereby may further interact with the surface of different cells, be adsorbed by macrophages, etc. According to this hypothesis, the triggering mechanism of the PE action depends on the mode of PE interaction with blood proteins as well as on the nature of complexes formed as a result of this interaction. Noteworthy that the “adhesiveness” of PE macromolecules within the composition of PEC which reflects the structural integrity of free sites of PE (loop-like structures, free ends) may differ considerably from that of the original macromolecule because in many cases, especially in the case of copolymers-polyampholytes, the complex formation is accompanied by the involvement of essential groups of PE in their interaction with proteins (selective fractionation of essential groups). Therefore, the mode of binding of individual PE and their complexes to cell surface membranes may be quite different. This difference obviates the need for a thorough analysis of mechanisms underlying the interaction of such protein-polymer complexes with the surface of immunocompetent cells.

This hypothesis is consistent with earlier reported data and with the results described herein. As it was mentioned above the length of the polymeric chain determines the composition of protein-polyelectrolyte complexes, and under identical conditions the number of bound protein globules attached to one PE macromolecule increases linearly in proportion to the elongation of the PE chain. As can be seen from Figure 120 there is a direct correlation between the adjuvant activity of PE and the number of protein globules bound to one PE macromolecule, i.e., the composition of the polycomplex. Similar results were obtained during the analysis of PE complexes with SDS and transient metal ions. PE or their complexes with low molecular weight ligands having a more unfolded structure and showing the ability to form nonstoichiometric complexes with proteins due to the high epitope density of their antigenic determinants “operate” as immunostimulators, whereas PE or PEC whose macromolecules have a compact structure and which form stoichiometric complexes in aqueous solutions are either inactive or produce a strong immunosuppressor effect. Consequently, the behavior of polymeric adjuvants in the blood flow depends on the supramolecular structure of their constituent macromolecules; the mode of the interaction with proteins (plasma or membrane proteins, etc.) predetermines the future fate of

PEC in the organism. Typical interactions of this kind are schematically presented in Figure 120.

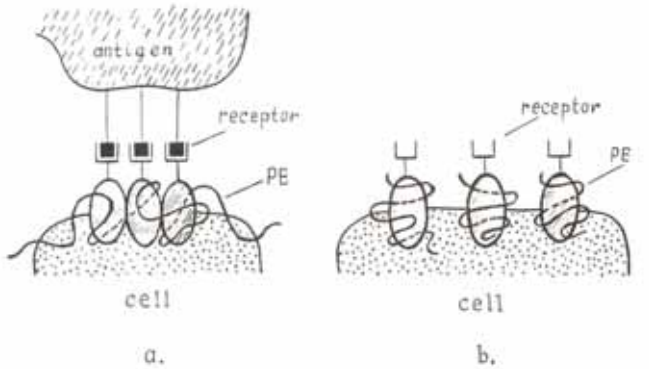



**Figure 120.** A schematic presentation of the interaction between linear (a, immunostimulators) and compact (b, immunosuppressor) macromolecules of PE having degree of polymerization with the antigen and the cell surface

During the formation of type "a" structures the local concentration of antigenic (or recaptures) determinants increases in parallel with the increase of the constants reflecting the multipoint interactions between the receptors and antigens; hence the high immunostimulating activity of such PE. Polyelectrolytes having a compact structure show a tendency to form complexes of the "b" type. In the latter case the initial local concentration of free receptors (or antigenic determinants) does not change, such PE either have no effect on the immune response or display an immunosuppressor effect by screening the receptor (or antigen) molecules.

These results provide compelling evidence in favour of the previously observed inhibiting effect on the immune response of polyampholytes containing both acidic (AA) and basic (MVP) groups [164]. Most probably, the suppressor effect of such polyampholytes reflects their structural peculiarities in aqueous solutions rather than a cooperative interaction as was believed earlier. Such polyampholytes have a compact structure, which is due to the intramolecular hydrophobic interactions of their methylpyridinium links. Their interactions with proteins result in the formation of stoichiometric complexes, which seems to be the main reason for their immunosuppressor activity.

Recently, the possibility of such migration was directly proved on the system, which models a cell suspension in aqueous medium (by Kabanov) [136,217-220]. The particles of practically monodispersed polystyrene latex of 0.5μm dimension, covered with chemically bound carboxyl groups (in average one COOH group per $25A^2$) served as cell models. The particles were labelled by means of chemical binding of fluoresceinisothiocyanate (FITC). PVP-$C_2H_5$ ($n \approx 10^3$) served as PE. Polycation units are capable of quenching of FITC luminescence. The curves of latex luminescence quenching by adding of PE at different pH are presented in Figure 121. The fact of quenching and the increase of quenching efficiency when the negative charge of latex particles is increased (with pH increase) indicate polycations adsorption on their surface. Ultra centrifuging with scanning in UV-adsorption range of PVP-$C_2H_5$ (250 nm) reveals that at pH=9 practically all PE are adsorbed on the latex particle surface. When a new portion of the same but nonlabelled latex is added to the system, the intensity of fluorescence sharply increases (Figure 122).

This unambiguously means that some fraction of PE macromolecules migrated to nonlabelled latex particles. It is probable that antigen-PE conjugate migrates, in a similar way, from cell to cell in search of antigen specific receptors. The search stops when the antigens find on B-lymphocyte surface the complementary receptors and bind with them. A minimum of free

energy corresponds to such state (Figure 123). In other words, the immunostimulant effect of PE is as if focused on certain cells. In the series of experiments PE conjugates with T-dependent (T,G-AL) polypeptide antigen of high specificity (polylysine graft copolymer with copolymer of alanine, glutamic acid and tyrosine [221,222,223] were used for mice of pure low-(CBA) and high-responder (C57/BL) strains These experiments revealed that the binding of antigen with synthetic PE not only increases the immune response to this antigen but also makes it T-independent and noncontrolled by Ir-genes [224].

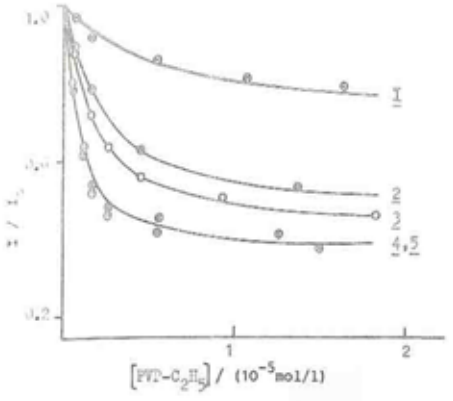



**Figure 121.** The dependencies of the fluorescence intensity of FITC-labelled carboxylated latex on PVP-$C_2H_5$ concentration, pH=1-6, 2-7, 3-8, 4-9, 5-10. Latex concentration c=1,3.$10^{-8}$ $cm^{-3}$; [$NA_2B_4O_7$]=0,01 mol/l, 25$^0$C

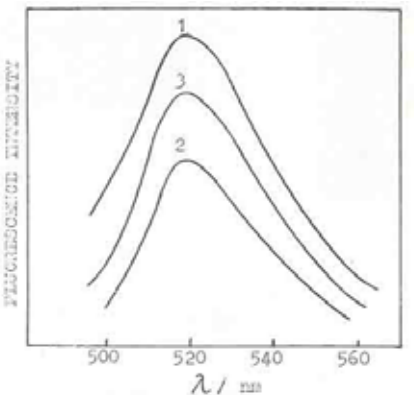



**Figure 122.** The fluorescence spectra of the model latex systems. I-FITC-labelled latex (c=1,3.$10^{-8}$ $cm^{-3}$), 2- FITC-labelled latex + PVP-$C_2H_5$ ($10^{-6}$ M), 3- FITC-labeled latex + PVP-$C_2H_5$ after adding of equal amount of nonlabelled latex (c=1,3.$10^{-8}$ $cm^{-3}$), pH=9. The turbidity spectra are subtracted

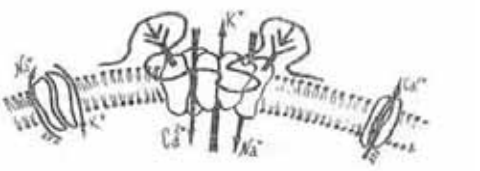


**Figure 123.** The hypothetic scheme of the interaction of an antigen-PE conjugate with B-lymphocyte membrane

### 5.2. Model Artificial Immunogens

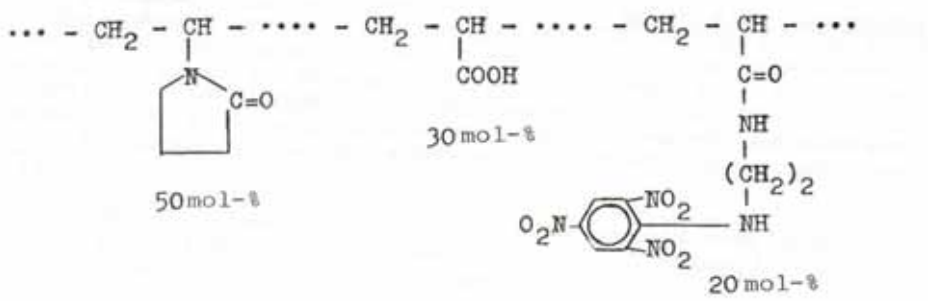
Nevertheless, B-lymphocyte activation by synthetic polyelectrolyte immunostimulants without help of T-lymphocytes is still not a solution of the whole problem. Firstly, the effect *in vivo* itself turns out to be not high enough: the number of antibody forming cells increases only by several times; secondly one and the same polyion acts nonspecifically: it can activate B-lymphocytes of various clones (polyclonal activation), i.e. inflicts the production of many mostly unnecessary antibody varieties [198,199].

Quite another result can be achieved if individual antigen or its active fragment–hapten is attached to the synthetic membrane active polyelectrolyte chain. Then, the strength of the T-independent immune response increases by dozens and hundreds of times. The immune response

becomes highly specific, i.e. antibodies against the antigen (or hapten) included into complex are produced.

Hapten or antigen attachment to polymeric immunostimulant can be achieved by means of either chemical or strong enough adsorbtion bonds, which cannot be destroyed in the organism for the time sufficient for switching on the immune system.

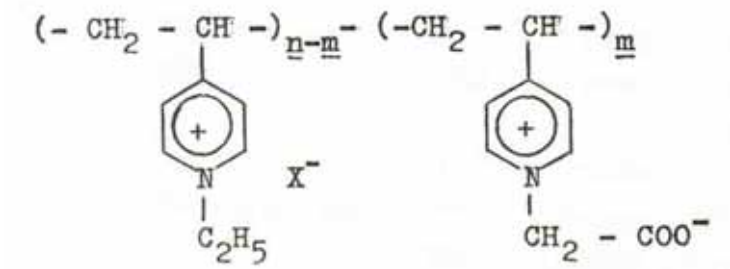
The first artificial antigen constructed on the basis of a synthetic PE is the electrostatic complex (EC) of trinitrophenol (TNP) with poly-2-methyl-5-vinylpyridine (PMVP) and covalent conjugates of TNP with copolymers of acrylic acid and N-vinylpyrrolidone [169,221]. The fragments of the artificial immunogen structures are presented on the following scheme:



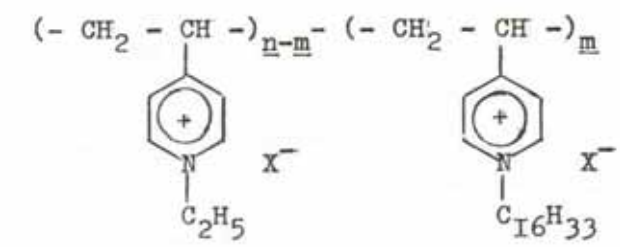

One distinctive feature of these antigens is that it not only elicits immune responses without the help of adjuvants but also is also able to stimulate the antibody production even in low responder athymic animals. It is evident that in contrast to TNP or its conjugates with protein carrier (bovine serum albumin). TNP-PE conjugate causes a very strong T-independent immune response, especially after secondary immunization. AFC specific to TNP are produced.

Recently, the possibility construction of such complete synthetic immunogens was demonstrated by the directly conjugation of steroid hormones (estradiol and progesterone), anticancer betuline and functionally polypeptides (Hepatite B surface antigen and Foot-and-Mouth Disease Virus VP1 protein epitopes) with different polyelectrolytes (see below).

Another group of artificial immunogens based on nonnatural PE carriers includes polycomplexes of protein antigens that are stabilized by cooperative electrostatic and hydrophobic interactions (Kabanov and Mustafaev, 1978) and covalent conjugates of PE with protein antigens (Kabanov and Mustafaev, 1978,1979). BSA covalently binding with copolymer:



where P=1000,m/n=0.4, or strong adsorbtion of BSA, BGG and some other proteins on copolymer:
where $P_n=10^3$,n/m = 0.1, resulted in the formation of the artificial antigens which cause much stronger immune response than the corresponding pure proteins.



It is noteworthy, that not only normal mice develop strong immune response (genetic strain C57BL/6(+/+)) but the thymusless as well (genetic etrain nude (nu/nu)). Consequently, the presence of linear PE fragments in artificial immunogen actually ensures the switching on of the immune reaction without the involvement of T-cells [26,158], i.e. these immunogens are able to stimulate the production of protein-specific antibodies all by themselves and "operate" independence of the control of the thymus or of the Ir-gene control of the immune response. In the series of experiments PE conjugates with T-dependent (T, G-AL) polypeptide antigen of high specificity (polylysine graft copolymer with copolymer of alanine, glutamic acid and tyrosine [222,223]) were used for mice of pure low-(CBA) and high-responder (C57/BL ) strains. These experiments revealed that the binding of antigen with synthetic PE not only increases the immune response to this antigen but also makes it T-independent and noncontrolled by Ir-genes [224].

The principle of conversion of relatively weak T-dependent antigens into very strong T-independent by their attachment to nonimmunogenic synthetic polyelectrolyte immunostimulants appears to be quite universal. It was confirmed by the examples of more than dozen of model, microbe protein and polysaccharide antigens (including BSA, BGG, OA, cancer protein antigen-alphafetoprotein, tuberculin, surface antigens of the influenza virus-hemagglutinin and neuraminidase, or the protein fraction of BCG (TPF), fraction F of the plague microbe, polysaccharide of salmonella O-antigen etc.) [9,9a,18,18a,26,39,85,105,118,158-186]. The antigen or hapten included into the polycomplex particle addresses the complex macromolecule as a whole, i.e. the artificial immunogen, to the antigen specific B-Lymphosite. The search of the B-lymphosite of the proper clone is realized by trial-and-error method by means of conjugate migration from sell to sell according to the mechanism of macromolecular substitution and exchange. This type of migration was discovered and studied by Kabanov [201,202] on the examples of interpolymer complexes and by Mustafaev on the examples of protein-polyelectrolyte complexes [38,60]. Recently, the possibility of such migration was directly proved on the system, which models a cell suspension in aqueous medium [218-220].

In this chapter we shall consider some physico-chemical criteria that are normally taken into consideration during the construction of artificial immunogens on the basis of PEC with special reference to the structural characteristics of PEC (stability, charge, size, conformation, etc.) as well as their immunological activity. A structural model of a polymer-subunit immunogen will also be described. These data shed additional light on the triggering mechanism of PEC. Besides they rationalize the selection of and outline the approaches to the directed synthesis of PEC with predetermined immunological activity.

**Highly immunogenic PEC.** The results of experiments aimed at the elucidation of immunogenic properties of soluble mixtures consisting of a model protein (bovine serum albumin, BSA) and PE, e.g., PVP and its derivatives, PAA, sodium polystyrene sulfonate (PSSNa) and their copolymers, are shown in Figure 124. It can be seen from these data that the immunogenic activity of the tested mixtures depends on the chemical nature of PE, although their immunostimulating activity is nearly identical.

What is the reason for this phenomenon? A detailed analysis of physico-chemical properties of PE-BSA mixtures revealed that the chemical structure of PE strongly affects their interaction with the protein antigen and, correspondingly, the stability of the polymer-protein

complexes formed thereby. It was found that under these conditions PVP acquires a weak positive charge and thus becomes unable to form complexes with BSA. The results of immunological studies suggest that the level of AFC production in BSA-PVP immunized mice is very low. However after the loading of PVB with lateral hydrophobic cetyl radicals (PVP-Rn) the former acquire the ability to form complexes with BSA.

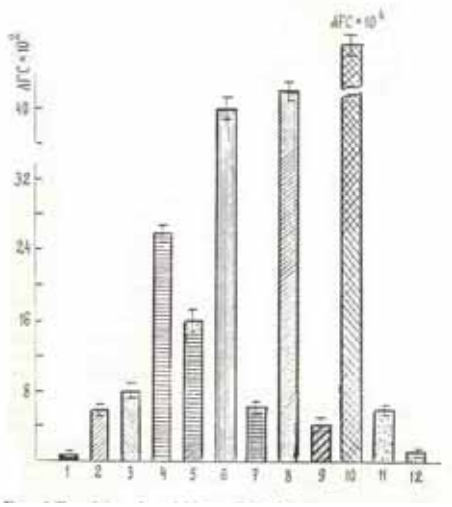



**Figure 124.** The relative values of BSA-specific AFC in the spleen cells of mice immunized with PE-BSA mixtures. 1- pure BSA; 2- PVP+BSA; 3- PVP-$R_{16}$+BSA; 4- (PVP-$R_2$+BSA) in $H_2O$; 5- (PVP-$R_2$+BSA) + 0,15 M NaCl; 6- PVP($R_2$,$R_{16}$) + BSA; 7- PVP($R_0$,$R_{ac}$) + BSA; 8- PVP($R_0$,$R_{ac}$) + BSA + carbodiimide; 9- PAA + BSA; 10- (PAA + BSA) + carbodiimide; 11- PSSNa + BSA; 12- CP(AA + MVP) + BSA.

The complex-forming capacity of PE molecules is different and depends on the number of alkyl radicals, ß, introduced into the PVP molecule. Polycations of PVP-Rn whose cetyl radicals are abundant enough to induce the formation of PVP-BSA complexes but which are insoluble in neutral aqueous media can form stable electrostatic and hydrophobic complexes with BSA in acid aqueous solutions (pH 4.3). However, at physiological values of the ionic strength and pH such complexes lose, to a certain extent, their stability: part of the protein molecules dissociate from the main complex to form an insoluble pellet, in which one polyionic chain corresponds to one protein globule (so-called stoichiometric complexes). With an increase in the ionic strength the newly formed water-soluble PSSNa-BSA complexes also lose part of their BSA molecules and at physiological concentrations of the low molecular weight salt acquires a stoichiometric composition. One may infer from these data that the same phenomenon will take place after injection of these complexes into the blood. Both complexes are characterized by a relatively low immunological activity, although they are more active than mechanical protein-PVP mixtures.

In solution PVP($R_2$)-BSA complex particles are stabilized due to the electrostatic interactions of oppositely charged PVP($R_2$) and BSA molecules. Under physiological conditions the EC formed in such mixtures are less stable and partly decomposed, as a result of which their immunogenicity markedly diminishes (Figure 124). In contrast with other systems, the formation of PVP($R_2$,$R_{16}$)-BSA complexes is provided for by cooperative electrostatic (salt) and hydrophobic interactions. Such complexes are rather stable and do not dissociate under physiological conditions. As can be seen from Figure 125, these complexes display a much higher immunogenic activity as compared with other known PEC. Speaking differently, among other

protein PE complexes used for immunization of animals the highest immunogenic activity is manifested by stable polyionic complexes that are resistant to physiological values of the ionic strength and pH. The structure of such complexes is depicted in Figure 125. The protein globules in each complex molecule seem to be in close contact with one another, being "entwined" by the polycationic carrier. Some hydrophobic cetyl radicals of PE are bound to the hydrophobic regions of the protein; while others interact with one another, thus promoting the stabilization of the overall structure. Fragments of the polycation containing no cetyl groups can form $H_2O$-accessible free loops or salt bridges with the negatively charged groups situated on the surface of the protein globules.

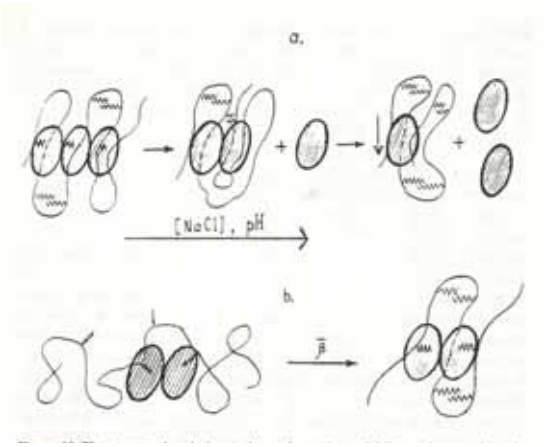



**Figure 125.** The structural and chemical transformations of PE-protein complexes in different reaction media. (a) BSA-PVP($R_0$,$R_{16}$) pH 4.3 (b) BSA-PVP($R_2$,$R_{16}$). For explanations see text

What is the functional significance of the covalent chemical bonds in this particular case? Its estimation is very important, both theoretically and practically, for all those whose ultimate goal is the construction of conjugates possessing maximal stability. In our studies this task was accomplished through the covalent attachment of the free carboxyl groups of PAA and 4-vinylpyridine copolymer with 4-vinyl-N-acetylpyridinium bromide [PVP($R_o$,$R_{ac}$)] to the amino groups of BSA via their activation by carbodiimide (Mustafaev *et al*., 1986):

BSA via their activation by carbodiimide (Mustafaev *et al.*, 1986):

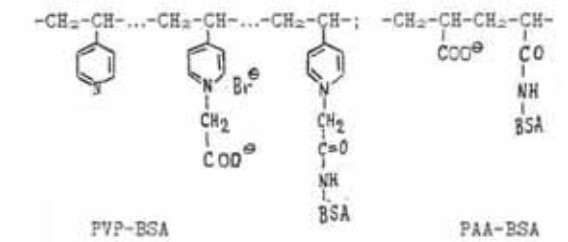



Figure 124 shows the results of immunological studies, in which the number of anti-BSA AFC in the spleens of mice immunized with polymer-protein conjugates is given relative to control. It can be seen from these data that the AFC number in the spleen cells of mice immunized with BSA covalently bound both to the polyacid and the polybases markedly exceeds that formed in the spleen cells of mice injected with an equal amount of the protein and PE that are chemically unrelated to each other.

The dependence of the mode of SRBC binding to PE on the immunogenicity of the PEC

formed thereby was especially apparent during the analysis of immunostimulating properties of PE able to form covalent complexes with corpuscular antigens (SRBC). In the previous chapter we emphasized the crucial role of the PAA chain length in the adjuvant activity of PE. In this case the immunostimulating activity of PE and SRBC was practically independent of the mode of their administration (separate or combined), being approximately at the same level. The stability of the PAA-SRBC complexes was provided for by the weak electrostatic ("mobile") bonds formed by the carboxylic groups of the polyacid and the corresponding sites situated on the heterofunctional surface of immunocompetent cells.

Naturally a question arises as to how the introduction of functionally important groups possessing the ability to form covalent bonds with SRBC influences the immunostimulating activity of PE.

Activation of PAA was induced by the routine carbodiimide method. This reaction is normally accompanied by the formation of anhydride bonds of the polyacid capable of forming covalent (amide) bonds with the amino groups of BSA as well as with the surface of SRBC. The relative values of AFC determined in the spleens of PAA-immunized mice at varying lengths of the PAA chain and at different numbers of their constituent anhydride bonds are shown in Table 10. These data suggest that the appearance in the polymeric molecule of anhydride bonds possessing the ability to form covalent complexes markedly increases the immunostimulating activity of PAA. With an increase in the number of such macromolecular bonds and, as a matter of consequence, with the elongation of the polymeric chain the adjuvant effect linearly increases. In this case the immunostimulating activity of PAA depends on whether it is injected alone or in combination with SRBC; its effect on immunogenesis is especially apparent after immunization of mice with ready-to-use solutions of covalent polymer-SRBC complexes.

**Table 11.** The effects of PAA and its anhydride derivatives on the AFC production in the spleen cells of SRBC-immunized mice. $AFC_{exp.}$ and $AFC_{contr}$ – AFC levels in experimental and control mice. $AFC_{contr}$ =4700. PAA and PAA* - original PAA and anhydride derivatives of PAA. β-number of anhydride bonds in the PAA molecule

| mode of injection of PAA and SRBC | PAA $AFC_{exp.}/AFC_{contr.}$ | | $AFC_{exp}/AFC_{contr}$ (PAA*) | | |
|---|---|---|---|---|---|
| | $\bar{P}_z = 570$ | $\bar{P}_z = 1100$ | $\bar{P}_z = 570$ $\beta = 10\%$ | $\beta = 20\%$ | $\bar{P}_z = 1100$ $\beta = 10\%$ |
| Separate | 2.0 | 3.30 | 4.0 | 5.0 | 4.8 |
| Combined | 1.4 | 2.35 | 3.0 | 7.25 | 9.2 |

These results suggest that under *in vivo* conditions the high stability of polymer-protein complexes is a necessary prerequisite for the manifestation of their high immunogenic activity.

In our further studies design ed to investigate the immunogenic activity of PE-protein complexes in more detail we used two types of stable complexes, namely, PVP($R_2$,$R_{I6}$)-BSA and the covalent conjugates, PAA-BSA and PVP-BSA. The kinetics of BSA-specific AFC accumulation in the spleen cells of mice immunized with PEC and CC constructed on the basis of polybases is shown in Figure 126. The experimental plots are characterized by the extreme; the peak of the immune response is observed on the 10th post-immunization day.

The dynamics of this reaction is irrelevant to the nature of the immunizing antigen and is manifested only in the increased amplitude of the immune response. In additional experimental series we studied the dose dependence of the immune response in the spleen cells of mice immunized with PVP($R_2$,$R_{I6}$)-BSA. The increase in the conjugate dose caused a proportional increase in the AFC level in mouse splenocytes.

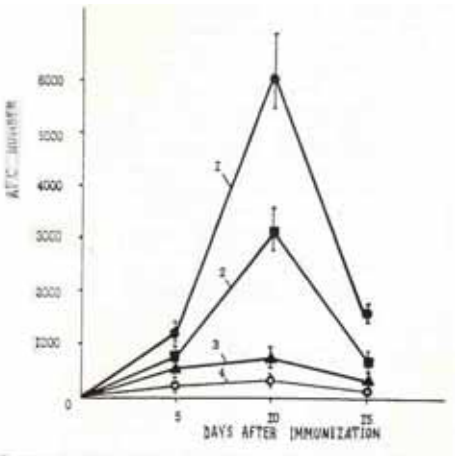



**Figure 126.** The kinetics of BSA-specific AFC accumulation in the spleen cells of mice immunized with BSA-PE complexes. 1-BSA-PVP($R_0$,$R_{ac}$); 2-BSA-PVP($R_2$,$R_{16}$); 3- BSA-PVP-$R_2$; 4-pure BSA. The BSA dose was the same in all cases

Specially designed experiments revealed that the AFC present in the spleens of mice immunized with such PEC were able to generate BSA-specific antibodies. The AFC accumulated in the spleens of PVP($R_2$,$R_{16}$)-BSA immunized mice could be detected only when BSA-SRBC (but not OVA-SRBC) was used as the test antigen. The AFC activity was fully eliminated by the BSA added to the agar.

Immunization of mice with BSA covalently bound to polymeric carriers having a different chemical composition (polyacid polyanions) also induced a high immune response whose magnitude was time-dependent (Table 12). Antibodies to BSA were detected in the blood sera of immunized animals already on the 4th postimmunization day; the peak of the immune response was observed on the 10th day with a subsequent decline on the 14th day. These AFC appeared to be specific towards BSA: an addition to the titration wells of pure BSA (final concentration *500,ug/ml)* caused a practically complete inhibition of the hemagglutinin reaction, whereas bovine γ-globulin (BGG) had no such effect. Therefore it seemed very enticing to investigate the effect of complete Freund's adjuvant (CFA) routinely used in immunological studies on the strength of the immune response elicited by a new artificial antigen. The conjugate was injected intraperitoneally with saline or in a water-in-oil emulsion with CFA at a 1:1 ratio (0.2 ml of the conjugate + 0.2 ml of CFA). The results of these experiments are depicted in Table 11. It can be seen from these data that the immunogenic activity of BSA-PAA conjugates markedly exceeded that of BSA + CFA mixtures, although the immune response to this antigen was not manifested immediately. A drastic increase in the immune response could be attained through a simultaneous administration of the conjugate and CFA: the AFC number was 30-40 times as high as that in BSA-PAA immunized mice and 100-130 times as high as that in BSA + CFA immunized mice. The latter finding points to a synergy of the "directed" effect of PAA and the stimulating effect of CFA on the BSA-specific immune response. It may be inferred from these data that the formation of ternary complexes in the conjugate-CFA system promotes the increase in the "adhesiveness" of the conjugate and thus stimulates the immune response to the protein antigen (see below).

It follows, therefore, that the combination of weak protein antigens with polymeric carriers markedly increases their immunogenicity irrespective of their chemical structure and the charge of their constituent macromolecules.

Similar results were obtained in experiments, in which PE were conjugated with other

proteins (model antigens, microbial and bacterial antigens). By illustration, complexes of BGG and the purified protein derivative of tuberculin (PPD) with quaternated PVP displayed a high immunogenic activity (Table 13). At the same time, complexes of the H-antigen of *Salmonella,* complete influenza antigen, meningococcal microbial B-polysaccharide and α-fetoprotein (oncofetal antigens) were also found to possess an exceedingly high immunological activity in comparison with the original antigens (see below). Hence, the observed phenomenon is unrelated to the nature of the protein antigen or the polymeric carrier and is more universal.

**Table 12.** The titers of BSA-specific antibodies in the blood sera of mice immunized with pure BSA., BSA-PAA, BSA-PAA in CFA + pure BSA and BSA-adjuvant. *-number of AFC formed in the spleen cells of mice immunized with BSA-PAA after addition of pure BSA (500μg/ml) to the titration plates

| preparation | antibody titers ($log_2$), M ± m | | | |
|---|---|---|---|---|
| | 4th day | 7th day | 10th day | 14th day |
| BSA | 0.5 ± 0.1 | 0.5 ± 0.12 | 0.5 ± 0.1 | 0.5 ± 0.12 |
| BSA + PAA | 0.5 ± 0.12 | 0.5 ± 0.15 | 1.0 ± 0.05 | 3.0 ± 0.14 |
| BSA-PAA | 0.5 ± 0.1 | 3.5 ± 0.20 | 5.0 ± 0.8 | 3.2 ± 0.15 |
| BSA-PAA* | 0.5 ± 0.1 | 0.2 ± 0.05 | 0.3 ± 0.05 | 0.3 ± 0.06 |
| BSA-PAA + CFA | — | 8.5 ± 0.19 | 11.0 ± 0.21 | 8.7 ± 0.20 |
| BSA + CFA | 2.0 ± 0.2 | 2.2 ± 0.15 | 3.0 ± 0.14 | 3.0 ± 0.15 |
| BSA – PAA | 0.5 ± 0.1 | 4.0 ± 0.30 | 5.0 ± 0.25 | 4.0 ± 0.25 |

**Table 13.** The AFC titers in the spleen cells of mice immunized with pure BSA, PPD and BGG-PPD complexes with quaternated Pvp loaded with lateral cetyl groups

| substance | AFC titers | number of animals |
|---|---|---|
| BGG | 5000 ± 5.35 | 18 |
| PVP($R_2$, $R_{16}$)-BGG | 100000 ± 15000 | 18 |
| PPD | 25 ± 5.0 | 18 |
| PVP($R_2$, $R_{16}$)-PPD | 25000 ± 2000 | 18 |

As above, pure PE carriers enhance the helper signal of T-Iymphocytes (Table 14), induce polyclonal activation of B-cells and promote the Ir-independence of the immune response to separately injected antigens. But can a protein conjugate with a polycation acquire the properties of a T-independent antigen? To answer this question we used as immunogens the polycationic complexes loaded with lateral hydrophobic groups combined with weak (BSA) and strong (BGG) protein antigens. The immunogenic activity of PVP($R_2$,$R_{I6}$)-BSA and PVP($R_2$,$R_{I6}$)-BGG complexes was studied on C57BL[6(+ / +)] mice as well as on athymic mice homozygous at the "nude" gene. The animals received intraperitoneal injections of the pure protein and an equal amount of the protein-PE complexes. The results of these studies are depicted in Table 14. It was found that in "nude" mice the immune response to pure BGG was much lower than in normal mice due to the T-helper deficiency: no specific AFC to pure BSA could be generated in these animals. However, injections of mice with BSA or BGG complexes with PE not only restored the original level of T-helper lymphocytes but also induced an additional synthesis of antigen-specific AFC.

**Table 14.** The effects of synthetic PE on antibody genesis in SRBC-immunized B-mice

| PE | $M_r$ | no. of animals | AFC number |
|---|---|---|---|
| — | — | 12 | 30 ± 10 |
| PAA | 80000 | 13 | 800 ± 150 |
| PVP | 50000 | 15 | 500 ± 80 |
| CP(AA-VPD) | 100000 | 9 | 4000 ± 500 |

Thus, conjugation of standard T-dependent protein antigens, both strong and weak ones, with T-independent polymeric stimulators allows one to obtain strong T-independent artificial immunogens and thus promotes the T-independence of the immune response to T-independent antigens within the composition of PEC. In practical terms, the ability of PEC based on protein antigens to elicit secondary (anamnestetic) responses to booster doses of the antigen and, correspondingly, to stimulate the immunological memory, presents special interest. In our studies mice were immunized with PVP($R_2$,$R_{I6}$)-BSA and, one month thereafter, with the pure protein antigen, after which the number of specific IgM- and IgG-synthesizing antibodies was counted in their spleens. The data depicted in Table 16 illustrate the levels of IgM- and IgG specific AFC determined in the spleens of experiment al mice on the 7th postimmunization day. After the primary (or secondary) immunization (M±Ip, $P \leq 0.05$) were by several orders of magnitude higher than in control animals. In these mice injections of the protein complex with the polycation stimulated the formation of stable immunological memory already after a single immunization, which made it possible to avoid the additional use of adjuvants.

**Table15.** The immunogenic activities of BSA. BGG and Their complexes with quaternated PVP loaded with lateral hydrophobie groups in the spleen cells of athymic and intact mice.

| genotype | immunogen | AFC | number of animals |
|---|---|---|---|
| C57BL/6(+/+) | BSA | 25 ± 10 | 5 |
| C57BL/6(+/+) | BSA-PVP($R_2$, $R_{16}$) | 2500 ± 400 | 6 |
| Nude (nu/nu) | BSA | — | 3 |
| Nude (nu/nu) | BSA-PVP($R_2$, $R_{16}$) | 600 ± 100 | 5 |
| C57BL/6(+/+) | BGG | 2500 ± 400 | 6 |
| C57BL/6(+/+) | BGG-PBP($R_2$, $R_{16}$) | 3500 ± 200 | 6 |
| Nude (nu/nu) | BGG | 100 ± 20 | 5 |
| Nude (nu/nu) | BGG-PVP($R_2$, $R_{16}$) | 20000 ± 2500 | 5 |

**Table 16.** The secondary immune response to BSA in mice immunized with pure BSA and a BSA complex with quaternated PVP loaded with lateral hydrophobic groups [PVP($R_2$, $R_{16}$)]

| immunization | | | AFC | |
|---|---|---|---|---|
| 1st | 2nd | number of animals | IgM | IgG |
| BSA | — | 18 | 23.5 ± 1 | 5.5 ± 2 |
| BSA | BSA | 11 | 20.0 ± 4 | 6.0 ± 2 |
| PVP($R_2$, $R_{16}$)-BSA | — | 15 | 3840 ± 470 | 3990 ± 300 |
| PVP($R_2$, $R_{16}$)-BSA | BSA | 14 | 5920 ± 720 | 7450 ± 680 |

**PEC with lowered immunogenicity**

As can be seen from Figure 125, in some cases tight binding of protein antigens to PE, e.g., PVP-

$R_n$ and PSSNa, causes no appreciable changes in their immunogenic activity despite rather a high immunostimulating activity of these polyelectrolyte carriers. Conjugation of BSA with CP(AA-MVP) also fails to increase the immunogenic activity; in this case CP eliminates the cooperative interactions of T- and B-Iymphocytes. Therefore in the next series of our experiments we carried out a detailed analysis of physico-chemical properties and immunological activities of several protein antigens with carbo-chain linear polyampholytes *(*Petrov *et al.,* 1982). These AA and MVP copolymers (CP) contained 66 (CP-1), 50 (CP-2) or 34 (CP-3) mol% links of MVP. Analysis of interactions of two model proteins, BSA and BGG, with these polyampholytes revealed that the tightness of their binding to each other depended critically on the number of MVP bonds, the most stable complexes being formed in the case of CP-1. The decisive role in the binding of likecharged molecules belongs to the hydrogen bonds and the nonpolar interactions of the protein globules with the hydrocarbon fragments of the polyampholyte chains. A typical structure of a CP-protein complex is shown in Figure 127.

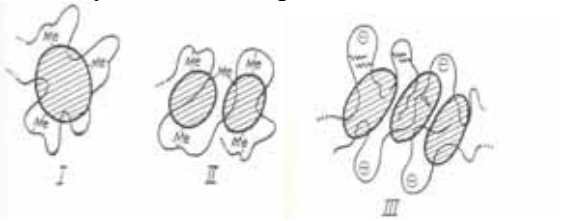


**Figure 127.** A schematic presentation of the structure of protein complexes with CP (III), PMC-1 (I) and PMC-2 (II). For explanations sec text.

The number of protein-specific AFC was determined after immunization of mice with the soluble polycomplexes, BSA-CP and BGG-CP. it appeared that the BSA complexes with these polyampholytes (Cp-1, CP-2 and CP-3) were practically devoid of immunogenic activity. In mice immunized with this PEC the levels of BSA-specific antibodies did not practically differ from "background" values.

Figure 128 shows the AFC titers in the spleens of mice immunized with pure BGG and its complexes with CP-1, CP-2 and CP-3. Within the composition of PEC BGG, it being a strong natural antigen, displays a higher immunogenic activity than in the free state. At the same time, in animals immunized with BGG-PEC mixtures having the same protein content the number of splenic AFC appeared to be different. For example, in the spleen cells of mice immunized with BGG + CP-3 the number of BGG-specific AFC was the same as in mice immunized with an equal dose of the pure protein antigen.

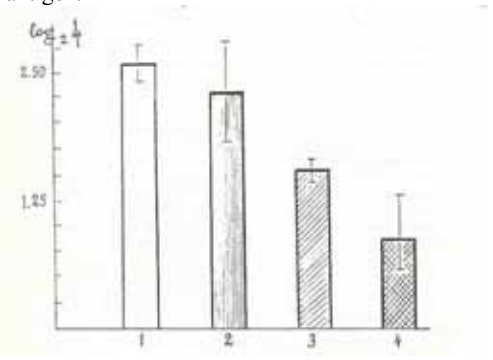



**Figure 128.** The immune response to BGG in the spleen cells of mice immunized with pure BGG (1), BGG + CP-3 (2). BGG + CP-2 (3) and BGG + CP-1 (4). Ordinate titers of BGG-specific AFC

Thus, MVP and AA copolymers, which form stable complexes with BGG suppress, whereas PE stimulate the immune response to the protein component of PEC. Marked inhibition of BGG-specific AFC production was seen when this polyampholyte was injected to mice together with *Clostridium perfringens* α-anatoxin [228]. It might be expected from these data that the observed differences in the immunogenic activity of proteins conjugated with polycations and polyampholytes is due to their different effects on the immunogenesis of polymeric carriers within the composition of PEC. In contrast with polycations possessing a high immunostimulating activity, copolymers of MVP and AA inhibit the cooperative interactions of T- and B-lymphocytes during the induction of immune responses to SRBC.

In this context the physico-chemical mechanism of this phenomenon can be interpreted in terms of the ability of polycations and polyampholytes to form complexes with structurally different proteins, which, in contrast with polyampholytes having a linear unfolded structure and a considerable chain length in aqueous solutions, possess rather a compact structure due to intramolecular hydrophobic interactions between their MVP bonds. This hypothesis has been open to criticism because the polyampholytes in question are heterogeneous both in respect of MMD and their chemical composition. Therefore, in our recent studies we used PMC of narrow fractions of a homopolymer (PVI), which differed in their molecular structure in aqueous media, i.e., PMC having a compact (PMC-1) and a crosslinked (more folded) conformation (PMC-2). As above, PMC-2 can form complexes both with BSA and BGG via transient metal ions, which play the role of crosslinking agents between the corresponding functional groups of the complex components. Noteworthy that PMC-1 exhibits an immunosuppressor, whereas PMC-2 - an immunostimulating activity (Mustafaev *et al.,* 1990). In neutral aqueous media these conjugates form complexes both with BSA and BGG at the expense of transient metal ions, which play the role of crosslinking agents between the appropriate functional groups of the complex components. In terms of their composition PMC-2 have an equimolar, where as PMC-2 - a nonstoichiometric structure (Figure 127, structures I and II) that reflects the differences in the structure of the original PMC. The ternary polycomplexes, BSA + PMC-1, BSA + PMC-2, BGG + PMC-1 and BGG + PMC-2 were used for immunization of mice, after which the levels of BSA- and BGG-specific AFC were determined in mouse spleens. The results of these experiments are depicted in Table 17. It can be seen from these data that the immunogenic activity of BSA + PMC-1 does not differ from background values, whereas that of BGG + PMC- is markedly decreased. The use of PMC-2 as carrier promoted a strong proteinspecific immune response in both systems.

It may thus be concluded that PMC-1 possessing a high immunosuppressor activity, rather a compact structure and the ability to form stable stoichiometric complexes with proteins suppresses the immune response, whereas PMC-2 which exhibits immunostimulating properties, has an unfolded (crosslinked) structure and the ability to form stable nonstoichiometric complexes strongly stimulates it.

It is very probable that PEC carrying a great number of protein molecules and having a larger molecular size in comparison with the original proteins circulate in the blood for longer periods of time and thus display a higher immunogenic activity. At the same time, PEC whose loose polymeric envelope surrounding the protein globule protects it from the deleterious effects of external factors, are characterized by a lowered immunogenicity.

These findings gave a strong impetus to our systemic studies aimed at the elucidation of relationships between the immunogenic activity of PEC and their chemical composition.

**Table 17.** The levels of protein-specific Afc in the spleen cells of mice immunized with pure BSA and BGG or their complexes with PVI (data from R. I. Gadzhiev), n.d. – not determined.

| immunogen | PMC dose, mg | protein dose, mg | number of animals | AFC |
|---|---|---|---|---|
| BSA | — | 0.5 | 16 | 35 |
| PVI-$Cu^{2+}$-BSA (PMC-1) + BSA | 0.01 | 0.5 | 18 | n.d. |
| BGG | — | 0.5 | 17 | 4400 ± 430 |
| PVI-$Cu^{2+}$-BGG (PMC-2 + BGG) | 0.01 | 0.5 | 16 | 340 ± 30 |
| PVI-$Cu^{2+}$-BSA (PMC-2 + BSA) | 1.0 | 0.4 | 18 | 5000 ± 740 |
| PVI-$Cu^{2+}$-BGG (PMC-2 + BGG) | 1.0 | 0.5 | 18 | 28000 ± 6500 |

### 5.3. The composition of PEC

We prepared three solutions of BSA + PAA at equal (w/w) concentrations of BSA and PAA: I- BSA + $(PAA)_1$, II - BSA + $(PAA)_2$ and III - BSA + $(PAA)_3$ having molecular masses ($M_w$) of 3, 40 and 80 kDa, respectively. All the solutions were prepared under conditions of the polyacid activation by carbodiimide by using the previously described procedure (Mustafaev *et al.,* 1986). Our experiments showed that in mice immunized with these mixtures AFC could be detected in the blood sera already on the 4th postimmunization day, the peak of the immune response being observed on the 10th day with a further decline on the 14th day. The titers of BSA-specific antibodies showed a great scatter in values depending on the solution used. The highest AFC titers were seen in mice immunized with complexes I and III, whereas in mice injected with solution II the titers of BSA-specific antibodies were minimal. It is noteworthy that the complexes used in this study differed from one another only by the molecular mass of the polyacid. Therefore the relative values of AFC and the titers of BSA-specific antibodies in the spleen cells of mice immunized with BSA-PAA mixtures (Figures 130A-130B) are given relative to $M_r$ of PAA. These data indicate that the AFC plots linearly depend on $M_r$ of PAA and are characterized by a minimum.

The dependence of antibody production on $M_r$ led us to suppose that the observed effect is due to the structural peculiarities of the BSA-PAA-carbodiimide complex molecules. A detailed physicochemical analysis of the experimental mixtures revealed that in all the cases the carboxyl groups of the newly formed conjugates were covalently bound to the amino groups of the protein via amide bonds (Mustafaev, 1986, 1989).

However, the mechanisms of the conjugate molecule formation in the above mixtures differ essentially from one another depending on the molecular mass of PAA. According to the ratio of the linear sizes of the polyacid and protein globules, such binding results in the formation of conjugates whose composition and, correspondingly, chemical structure also show substantial differences. Figure 130B shows the composition of conjugates obtained at different values of $M_w$ for PAA. At $M_{w(PAA)} \geq 40$ kDa the number of bound BSA globules increases linearly with the increase in the length of the PAA chain. In the case of $(PAA)_2$ the conjugate has a stoichiometric composition (1:1) (structure II), where as that of $(PAA)_3$ is nonstoichiometric, for one macromolecule of PAA appears to be bound to three molecules of BSA (structure I). In the case of the PAA complex with a relatively low molecular weight polymer the situation is quite different. At $M_w$ = 3 kDa the mode of binding changes: the protein globules now play the role of the carrier. This may lead to the formation of two structural types: i) conjugates formed by interacting BSA globules that are crosslinked by multiple short chains of the linear polyacid

(structure IIIb) and, ii) conjugates in which each protein globule is bound to several PAA molecules. Such conjugates are not crosslinked and form a "hedgehog"-like branched structure (IIIa).

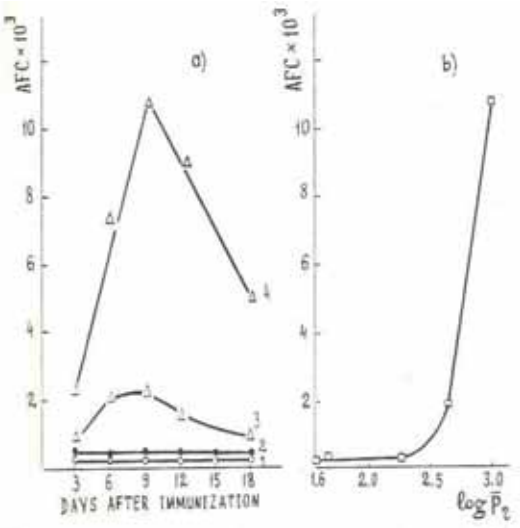



**Figure 129.** The dynamics of AFC production in the spleen cells of mice immunized with covalent conjugates (BSA-PAA) at different lengths of the PAA chain (n). 1- Pure BSA; 2- BSA-PAA (n=43); 3- BSA-PAA (n=550); 4- BSA-PAA (n=1140). At n=43 the structure of BSA-PAA was the same as that of IIIa (Figure 129). (b) The dependence of the AFC number on the degree of polymerization (β) of PAA within the composition of the PAA-BSA conjugate

A comparative study of BSA-PAA mixtures and analysis of their immunogenic activity revealed that the conjugates "enriched" with protein molecules exhibit the highest immunogenic activity (Figure 130A). The high epitope density of the protein antigens within the composition of the polymeric carrier confers on such PEC a high immunogenic activity. As already mentioned, the conjugates having an equimolar composition are characterized by the lowest immunogenicity. However, in these studies we succeeded in obtaining conjugates of different composition by changing the molecular mass of PE. It follows, therefore, that the above conclusion postulating a relationship between the composition of CC and their immunogenic activity may be rather premature.

Therefore in the next series of experiments we performed a chemical synthesis of PEC in which one macromolecule of the polymeric adjuvant irrespective of its molecular mass was attached to an equimolar amount of the protein antigen, after which the immunogenic activity of the complexes was estimated (Mustafaev and Norimov, 1988). The structure of such synthetic PEC is shown in Figure 131.

In our work we used polycations carrying lateral cetyl radicals. Each macromolecule irrespective of its molecular mass was bound to no more than two protein molecules. In all the eases the binding was achieved through the formation of a multipoint system of electrostatic salt contacts stabilize d by the embedment of hydrophobic cetyl radicals into the hydrophobic regions of the protein globules, thus promoting the stability of the complexes at physiological values of the ionic strength.

The water-soluble complexes thus prepared were used for immunization of intact mice, after which the levels of protein-specific AFC were determined in their spleens. As can be seen from Table 18, in all the eases studied the strength of the immune response increased significantly

in comparison with the control group. The relative values of AFC did not practically depend on the molecular mass of the polycationic carrier, being approximately at the same level. These findings led us to conclude that the immunogenicity of a polycomplex is correlated with the number of bound antigenic molecules, i.e., with their epitope density, and (at least within the studied range of polymerization degrees) it does not depend on the length of the polymeric chain.

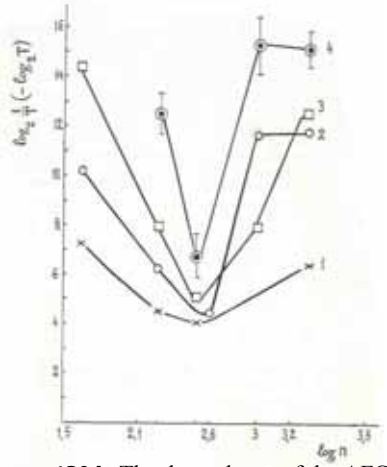


**Figure 130A.** The dependence of the AFC titers ($-\log_2 T$) in the blood sera of mice immunized with PAA-BSA conjugates on the length of the polymeric chain (n). 1-primary immune response (13th day); 2, 3, 4-secondary immune response: 7th day (2), 11th day (3) and 10th day (4). At n = 43 the structure of CC was the same as that of IIIb

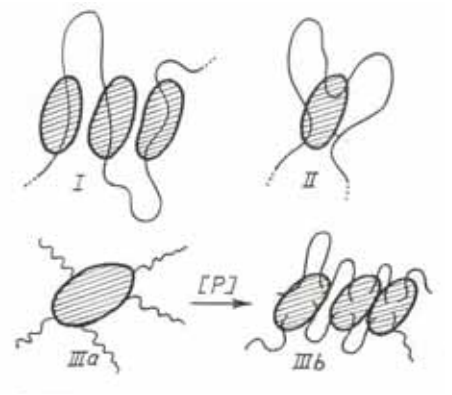



**Figure 130B.** A schematic presentation of the structure of covalent conjugates (PAA-BSA) at different lengths of the polymeric chain (degree of polymerization), n: 1140 (I), 550 (II) and 43 (III). At n=43 two types of structures were formed depending on the BSA/PAA ratio. For explanations see text

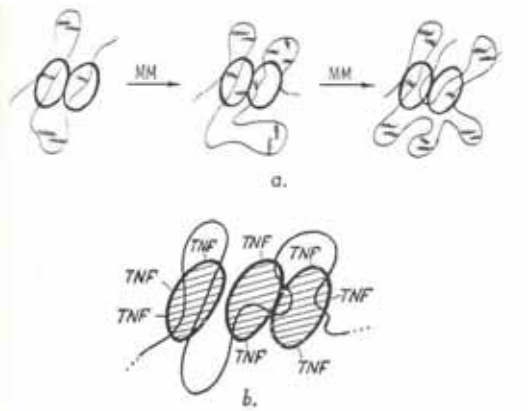



**Figure 131.** The dependence of the structure of water-soluble complexes of BSA with PVP($R_2$,$R_{16}$) on the degree of PE polymerization (n): 103 (a), 2.15 x$10^3$ (b) and 7.4 x $10^3$ (c). Degree of quaternization by: cetyl bromide-7-9 mol%, ethyl bromide- 90 mol%. (b) A schematic presentation of ternary water-soluble complexes, PAA-(BSA-TNP)

**Table 18.** The levels of protein-specific AFC in the spleen cells of mice immunized with pure BSA and BSA complexes with quaternated PVP loaded with lateral hydrophobic groups at different degrees of polymerization (P) of PE

| immunogen | P, $10^3$ | AFC | number of animals |
|---|---|---|---|
| BSA | — | 25 ± 5.0 | 18 |
| PVP($R_2$, $R_{16}$)-BSA | 1.0 | 2800 ± 250 | 17 |
| | 2.15 | 2400 ± 200 | 16 |
| | 4.5 | 1900 ± 180 | 18 |
| | 7.5 | 2000 ± 222 | 16 |
| | 12.0 | 2100 ± 200 | 18 |

The dependence of the composition of PEC on their immunogenic activity becomes especially apparent in the cage of microbial antigens, e.g., B-polysaccharide (PS) that represents a purified meningococcal antigen. The AFC values and antibody titers relative to the PS/PE ratio (w/w) are given in Table 19. It can be seen from these data that the highest titers of protein-specific antibodies (including AFC) are characteristic of PS-enriched complexes.

**Table 19.** The immunogenic activity of the pure meningococcal B-prolysaccharide and the antigen-polymer complex at different w/w ratios of the antigen-polymer mixture. PS – polysaccharide. PE concentration was varied at a constant concentration of PS

| PS/PVP($R_2$), w/w | AFC | antibody titers ($log_2$) |
|---|---|---|
| Control (PS) | 30 ± 5 | 0.3 ± 0.05 |
| 2 | 80 ± 7 | 0.6 ± 0.1 |
| 5 | 150 ± 11 | 1.4 ± 0.12 |
| 7 | 170 ± 15 | 1.2 ± 0.1 |

Thus, the composition of artificial thymus-independent antigens is another factor, which determines, to a large extent, their immunological activity.

One necessary prerequisite to the induction of high immune responses is the binding of more than one molecule of the protein antigen to one macromolecule of the polymeric carrier. However, the role of bound protein molecules and their number in the immunogenic activity of protein-polymer conjugates will finally be clarified only after the chemical synthesis of conjugates whose polymeric chains carry different numbers of protein molecules at an equal degree of polymerization (macromolecular size) and compactization of their coils. Studies in the field are currently under way. It is conceivable, therefore, that the strength of the immune response to haptens included in the composition of PEC depends on their epitope density.

The first artificial antigen, MVP-TNP, by reason of its low solubility in aqueous media cannot be regarded as a convenient model in physico-chemical studies. The impossibility to control the composition and, correspondingly, the epitope density of the hapten determinants of the polymeric carrier is a considerable obstacle to the wide-scale application of such complexes in immunological studies. In complete contrast, polymer-protein complexes are highly soluble in neutral aqueous media; the composition and size of their constituent molecules are readily controllable. The protein fragment of PEC retains both its, native conformation and accessibility to other molecules. Therefore in further studies we prepared water-soluble PEC with a different content of TNP and tested these complexes for immunological activity towards the immobilized hapten (Mustafaev *et al.,* 1989). TNP was at first bound to BSA in order to achieve the incorporation of the TNP-groups into PEC, after which the TNP-BSA conjugates were mixed with PAA in neutral aqueous solutions. Under these conditions both PAA and pure BSA acquired

the like negative charge, which made impossible their further involvement in the complex formation (Mustafaev, 1981). The situation was quite different when aqueous solutions of TNP-BSA were mixed with PAA. BSA, which contains only five TNP-groups, was also unable to interact with the polyacid to form stable PEC. However, a further increase in the number of TNP groups led to the formation of stable ternary water-soluble complexes on the BSA molecule, PAA($TNP_{17}$-BSA) and PAA($TNP_{26}$-BSA).

The structure of these complexes carrying hapten groups is schematically presented in Figure 131; immunological data are shown in Figure 132. After a single immunization of mice with TNP-BSA conjugates the latter were unable to stimulate the production of TNP-specific antibodies: the immune response to them was manifested only in the presence of CFA, whereas the relative values of AFC were increased 3.5-5-fold.

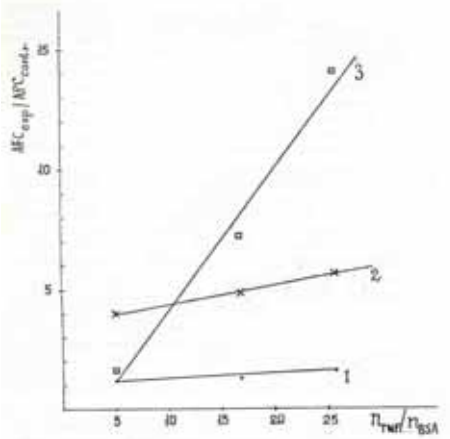



**Figure 132.** The dependence of the relative values of TNP-specific AFC ($AFC_{exp}/AFC_{contr}$) (ordinate) after immunization of mice with TNP-BSA (1), (TNP-BSA). CFA (2) and (TNP-BSA)-PAA (3) on the number of TNP groups in the conjugate mixtures (abscissa). $AFC_{exp}$ and $AFC_{contr}$-AFC levels in the experimental and control groups, respectively.

It should be noted that in this cage the strength of the immune response did not practically depend on the number of TNP molecules bound to the protein carrier. However, in mice immunized with PAA- ($TNP_x$-BSA a strong immune response to TNP was elicited: its magnitude varied considerably depending on the number of hapten groups in the PEC molecule. The relative values of AFC increased linearly in parallel with the increase in the number of TNP groups. At x = 5, as above, neither PAA nor TNP-BSA, being immunologically inert, were able to form such complexes. In complete contrast, in their mixtures an augmented synthesis of protein-specific antibodies concomitant with the formation of stable complexes took place.

It follows from these data that immobilization of the hapten molecules on stable water-soluble PEC markedly increases (10-15 fold) the immune response to the given hapten. In contrast with water-in-oil mixtures (e.g., CFA), in PAA-(TNP-BSA) one macromolecule of PAA binds several molecules of the TNP-BSA conjugate, as a result of which the epitope density of the regularly organize d TNP groups within the complex molecule linearly increases which, in turn, causes a linear increase of the AFC titers relative to the number of the TNP groups included into PEC.

### 5.4.Immunogenicity of PEC in the presence of competitive PE

Within living organisms polycomplexes of protein antigens can interact with blood proteins, cell

surface as well as with other strongly charged macromolecules, such as nucleic acids and acid polysaccharides that are present in large amounts in the animal blood. It seemed, therefore, important to investigate the immunogenic activity of polymer-antigen complexes in the presence of competitive PE.

To this end we selected two types of artificial antigens [168,174,175], one of which represented an electrostatic complex (EC) of BSA with poly-4-vinyl-N-ethylpyridinium bromide, whereas the other one was a covalent conjugate (CC) of BSA with carboxymethylated PVP. These studies revealed that EC and CC differently interacted with heparin, a polyanion characterized by a high charge density *in vitro* (Mustafaev and Kabanov, 1980). An addition of heparin to EC led to the complete displacement of BSA, which remained in solution, whereas the stoichiometric heparin-PE complex formed an insoluble pellet (Figure 133).

The situation was quite different when heparin was added to CC. In this case the polyanion did not displace BSA from the complex, because the covalent bonds formed between the protein and PE prevented the macromolecular substitution but facilitated the formation of a soluble complex between heparin and CC.

Immunological studies were conducted on mice injected with definite doses of heparin immediately after their immunization with EC or CC or 30 min, 2 hours and 24 hours thereafter. The AFC titers were determined at the peak of the immune response, i.e., on the 9th post-injection day.

The relative values of AFC in the spleen cells of mice measured at different periods after heparin injection are shown in Table 19, it can be see n from the se data that heparin injected immediately after EC or 30 min after immunization of animals fully prevented the immune response to the artificial antigen. In this way heparin "encompassed" EC within the organism and caused its destruction by binding to the polycation and thus displacing the protein before the immune response could be triggered on. Being injected 2 hours after immunization, heparin was still able to elicit the immune response, although in this case its amplitude was lower than in the control group (in the absence of heparin). Heparin injected 24 hours after EC had no effect: the magnitude of the immune response was the same as in the control group.

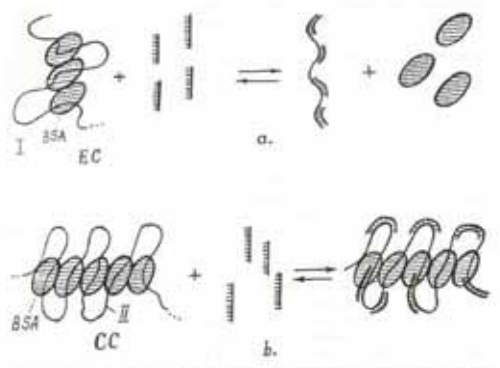



**Figure 133.** A schematic presentation of EC (a) and CC (b) interactions with heparin (c). For explanatations see text

In animals immunized with CC heparin did not practically affect the strength of the immune response irrespective of the time of its administration. It is noteworthy that in this case heparin was unable to prevent the CC interaction with the membranes of appropriate immunocompetent cells even if it could "encompass" the conjugate within the organism.

These data are consistent with the behavior of EC and CC in the presence of heparin under *in vitro* conditions. The results of these studies present substantial interest from two

viewpoints. First, they provide a perfect model of exchange reactions occurring in living organisms. Second, they make it possible to calculate the minimal time needed for the immune response to an artificial T-independent antigen to be triggered on. Both considerations are very important for the practical solution of many fundamental problems of modern day immunology.

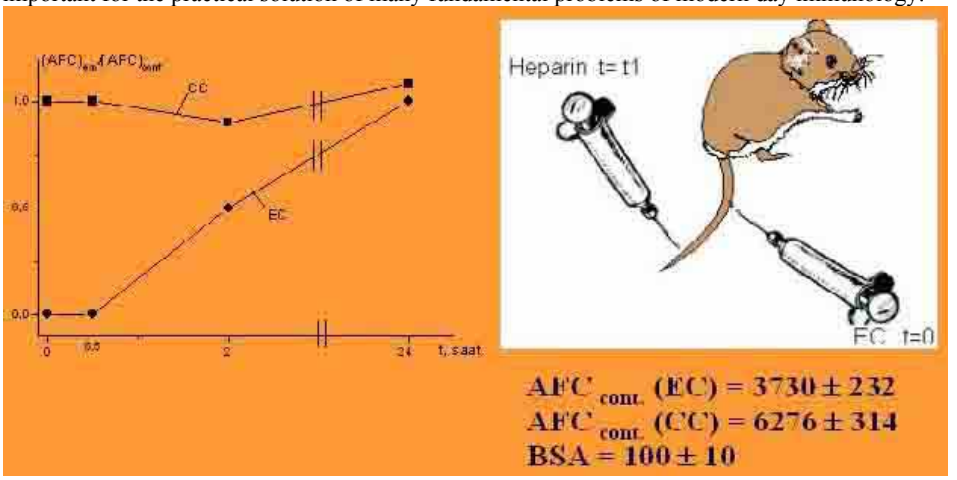



**Figure 134.** The relative values of AFC ($AFC_{exp.}/AFC_{contr.}$) in the spleen cells of mice immunized with EC or CC at different times after heparin injection. -$AFC_{contr.}$ After immunization of mice with EC and CC were 3730±232 and 6276±314, respectively.

**The loop-like structure and "adhesiveness" of PEC.** Needless to say that the further fate of PEC, CC or free PE entering the organism depends, primarily, on the mode of their interaction with plasma proteins, cells and other charged constituents of the blood. Therefore model studies of antigen interactions with proteins and cells may provide valuable information for all those who are engaged in the study of the structure and immunological activity of such antigens. These studies are all the more important, because they allow one to discover previously unknown factors, which play a crucial role in the manifestation of the high immunogenic activity of artificial immunogens.

It is known that macromolecules of polymer-protein complexes contain flexible free sites of PE (Ioop-like structures, free ends) that are not directly involved in the interaction with the protein globules. These free sites form an extensive hydrophobic area along the surface of the PEC molecule, which, in turn, promotes their high solubility. In solutions containing other charged macromolecules the physicochemical properties of PEC (interactions with proteins, surface adsorption, etc.) depend on their chemical nature as well as on the structure of their free polymeric fragments.

Data from physico-chemical analyses of PEC interactions with other proteins suggest that according to the mode of their binding PEC can be divided into two groups. The first group includes PEC, in which the free sites of the polymeric carrier can effectively bind protein molecules in model systems in the presence of blood serum proteins. In this case the binding is provided for by cooperative electrostatic or hydrophobic nonpolar interactions; their combination promotes a high level of PEC binding to the protein. As a result of PEC interactions with polycations the net positive charge of the PEC molecules decreases; a further rise in the protein concentration leads to their aggregation (Figure 135). Noteworthy that such PEC displays a high immunogenic activity. This group of PEC also comprises complexes (or conjugates) with negatively charged polymeric carriers, including protein conjugates with a polyacid (e.g., PAA).

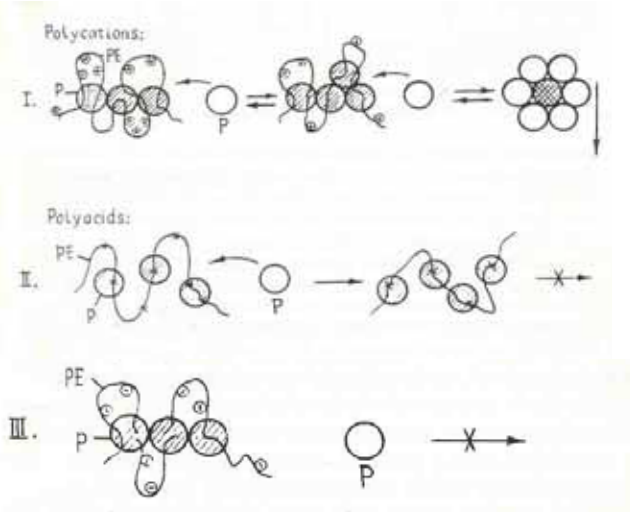



**Figure 135.** A schematic presentation of the interaction between polymer-protein complexes (conjugates) having a characteristic composition with free addional protein molecules. For explanations see text.

Such PEC is also endowed with the ability to bind additional protein molecules via multipoint electrostatic interactions between their carboxyl groups situated in the loops of the polycomplex. However, in contrast with PE complexes with polycations, under these conditions the rise in the protein concentration is unaccompanied by the aggregation of the conjugate molecules or the formation of an insoluble pellet. The excess protein remains in solution in an unbound state (Figure 135, structure II). The immunogenic activity of such complexes is also rather high.

The second group comprises PE, in which the free sites of the polymeric carrier are devoid of re active (complex-forming) functional groups and are thus unable to bin d additional protein molecules. Protein complexes with linear polyampholytes provide an illustrative example of such PEC. The critical role in the complex formation belongs to hydrogen bonds and nonpolar interactions between the methylpyridinium links and the negatively charged protein globules bearing the like negative charge. This leads to the formation of strands between the interacting protein globules that are linked together by linear polyampholyte chains. The negatively charged functional groups situated in the free sites of such polyampholytes are not directly involved in the protein binding, as a result of which the complex particles acquire a surplus negative charge. For this very reason such PEC are unable to form complexes with extrinsic proteins. The immunological data suggest also that these PEC differ from those described in the previous chapters in that they display a much lower immunogenic activity in comparison with the original highly immunogenic protein antigen.

Hence a question arises as to how the "closure" of the loops in the structure of PEC induced by protein antigens affects the immunogenicity of these complexes. An answer to this question came from the analysis of PEC constructed on the basis of polycations and a model protein (fraction $F_1$ of the plague microbe [229]. In contrast with serumal proteins, protein $F_1$ shows the ability in .a strong intermolecular association in aqueous solutions. For this very reason the mechanism of its, binding to polycations differs essentially from those of BSA, BGG" etc. The initial step of the complex formation in the $F_1$-PE mixture is the uneven distribution of protein molecules between the adsorbing polycations. Such a system is made up of two fractions: a free polycation and the Ft-PE complex. A similar mechanism of the complex formation was

observed during the interaction of PE with the, protein antigen of the plague microbe having rather a high molecular mass (ca. 1.2 x $10^6$*)* (Figure 137, scheme III). Quite a different situation was seen after a further increase in the $M_r$ of the antigen. By reason of the strong ability of $F_1$ to associate after its addition to the mixture at a concentration, which exceeded a certain critical level, i.e., the protein/PE ratio, the complex formation was accompanied by the binding of the protein molecules to the already formed molecules of PEC (but not to free PE). Such a mechanism promoted the binding of additional $F_1$ molecules in the free loops of PE within PEC as well as the formation of molecular associate s of limited solubility. The structure of such PEC is characterized by the lack of free polycationic sites (Figure 135). Immunological studies revealed that the highest immune responses in delayed type hypersensitivity (DTH) and phytohemagglutinin (PHA) tests were elicited after immunization of animals with mixtures composed of a free polycation and a PEC having a loop-like structure. With an increase in the $F_1$ concentration in the solution of the polymeric carrier the free sites (loops) disappeared, and the mixtures containing such PEC became inactive. The stimulation indexes (SI) were in both cases dose to unity.

Immunization of animals with PVP($R_2$,$R_{I6}$)-OVA provides an illustrative example of how the free sites of PE influence the immunogenicity of PEC. The results of immunological studies in which mice were immunized with a single dose of water-soluble PEC-OVA complexes prepared at different [OVA]/[PE] molar ratios at a constant concentration of OVA and at varying concentrations of PE are listed in Table 20. It can be seen from these data that the titers of IgE antibodies exceeded the control values in those cases when PEC contained free sites of PE noninvolved in the binding with the protein ($n_{OVA}/n_{PE} \leq 4$) *(*Mustafaev *et al.,* 1990). An increase in the protein concentration in the PE-OVA mixture caused a drastic reduction of the number of IgE AFC down to the control level.

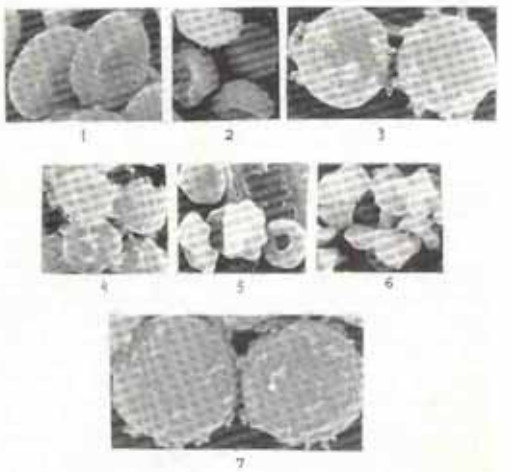



**Figure 136.** A scanning electron microscopic view of normal SRBC (1, x 6300); SRBC + PAA (2, x 2400); SRBC + PVP($R_2$,$R_{16}$) (3, x 10800); SRBC + BSA- PVP($R_2$,$R_{16}$) (4, x 10800); SRBC + BGG-CP (AA-MVP) (6, x 4200) and SRBC + (PAA-BSA) (7, x 1500). PVP($R_2$,$R_{16}$) (5, x 12800) was added to SRBC + BGG. [230].

The different "adhesiveness" of PEC molecules in the protein globules, which was found to depend on the chemical nature of the polymeric carrier, was also manifested during their interaction with SRBC and spleen cells (SC) of intact mice [230,231]. Figure 136 shows a scanning electron micrograph of normal and washed from contaminant proteins SRBC and their mixtures with PE, PEC and SC. It can be seen from the se data that the shape and size of SRBC

changed considerably after the addition of PE and BSA-PVP($R_2$,$R_{I6}$) which testifies to a strong interaction of different cell fragments with PE. One can visualize large associate s formed by surface proteins of SRBC and PE. After mixing of PE with SRBC in the presence of a specially added protein the mode of PE binding to SRBC did not change: the characteristic alterations in the shape and structure of the cell surface can be seen in the micrographs.

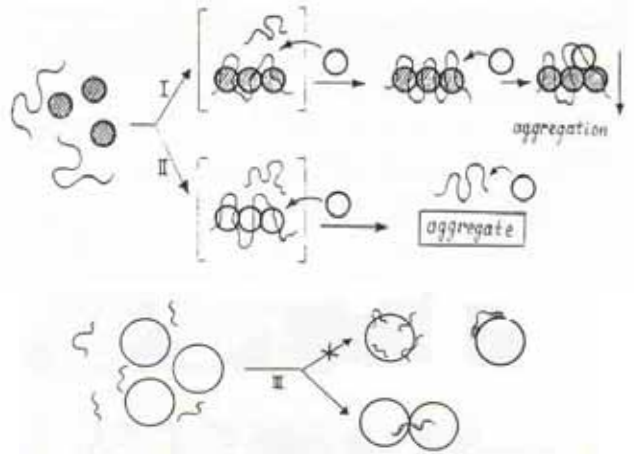



**Figure 137.** A schematic presentation of the PE binding to blood serum proteins (I) and plague microbe fractions $F_1$: 120 kDa (II) and 1200 kDa (III)

In contrast with pure PE, the interaction of PE molecules with SRBC was accompanied by the appearance on the cell surface of a large number of vesicles and finger-like projections called microvilli (cones). Incubation of polycations and their protein complexes with SRBC resulted in the clusterization (aggregation) of these molecules as a result of nonspecific interactions of SRBC with the polycation. In contrast with PE, incubation of SRBC with the negatively charged PAA, CP(AA-MVP--BGG and PAA-BSA did not induce any appreciable alterations in SRBC, although there were some changes in the cell shape. The surface of SRBC remained smooth; there were no signs of intercellular clusterization.

**Table 20.** The titers of OVA-specific IgE antibodies after a single immunization of mice with pure OVA or OVA-PEC at different values of the [OVA]/[PEC] ratio (Popov, unpublished data).
* - The AFC titer for the IgE response to pure OVA was 4.0.

| | IgE titers ($\log_2$ 1/T) | |
|---|---|---|
| $\eta_{OVA}/\eta_{PE}$ | PVP($R_2$, $R_{16}$)-OVA* $\beta = 5$ mol% | PVP($R_2$, $R_{16}$)-OVA $\beta = 7$ mol% |
| 2.0 | 6.6 | 7.8 |
| 3.0 | 5.6 | 5.5 |
| 4.0 | 4.0 | 5.1 |
| 5.0 | 3.4 | 4.0 |
| 6.0 | 4.0 | 3.5 |
| 8.0 | 3.0 | 4.8 |

The binding of PEC to the SC surface was estimated by three independent methods [230]. At first isolated SC ($10^7$ cells/ml) were incubated with radiolabelled OVA in a serum-free medium 199 in the presence of fluorescent isothioeyanate (OV A-F) or its complexes with

PVP($R_2$,$R_{I6}$) (PEC-1) and CP(AA-MVP) (PEC-2). The percent content of the fluorescent cells and the mean values of the fluorescent intensity were measured with the help of a flow cytofluorimeter. In the second case SC ($10^7$ cells) were incubated with aqueous solutions of OVA or OVA-PEC for 10 min, after which SC were sedimented by centrifugation. Matrix solutions obtained after incubation with SC were analyzed by spectrophotometric methods. In the third series SC were incubated with $^{125}$I-0VA or its complexes with PEC (100 μg/ml of OVA) in the medium 199. The radioactivity of the cell samples after their incubation and subsequent washing was measured on a specially designed counter. The degree of clusterization (aggregation) of SC in the presence of OVA or PVA-PEC was assessed by light microscopy and expressed as the number of cell aggregates (three or more cells) per 100 single and aggregated SC.

The results of these experiments are listed in Table 21. It can be seen from these data that in the case of OVA-PE the concentration of the fluorescent cells reached the maximum (> 80%) already during the first 5-12 minutes of incubation of the PEC-SC mixtures and remained at this level throughout the observation period. At the same time, the percent adsorption of the pure protein and its complex with the polyampholyte (CP) was markedly decreased, being about 10-15%. Similar results were obtained during the analysis of mixtures by radioisotopic and spectrophotometric techniques (Table 22).

**Table 21.** The dependence of the per cent content of fluorescent spleen cells on the time of their incubation with OVA-PE complexes

| mixture | incubation time, min 5 | 15 | 60 | 120 |
|---|---|---|---|---|
| SC + [PVP($R_2$, $R_{16}$)-OVA] | 68 | 85 | 90 | 70 |
| SC + [CP(AA-MVP)-OVA] | 5 | 10 | 22 | 20 |
| SC + OVA | 4 | 5 | 7 | 8 |

Thus, the adsorptive capacity of OVA-PEC on the SC surface markedly exceeds that of OVA complexes with the linear polyampholyte. Stipulating that the presence on the cell surface of OVA-specific antibodies or receptors can be neglected, it may be assumed that the observed difference in the PEC binding is due to their physico-chemical peculiarities. The high adsorptive capacity of PEC-1 can be explained by the presence in its composition of positively charged free sites (loop-like structures, free ends) of PE containing fragments of hydrophobic radicals able to interact in a nonpolar fashion with cell membranes. The complex formation between OVA and polyampholytes is due to the nonpolar interactions (very probably, "forced" ion-to-ion contacts) of OVA molecules with the hydrocarbon fragments (methylvinylpyridinium links, MVP bonds) of the CP chain, eventually resulting in partial "fractionation" of the functional groups depending on the composition of PE.

**Table 22.** The adsorptive capacity of pure OVA and OVA-PEC measured by different methods. *- OVA concentration, 100μg/ml. $C_{PEC}$ – concentration of adsorbed OVA within the composition of PEC; $C_{OVA}$ – concentration of adsorbed OVA

| immunogen | spectrophotometry (% of maximum) | radiolabeling ($C_{PEC}/C_{OVA}$) |
|---|---|---|
| OVA* | 55 | — |
| PVP($R_2$, $R_{16}$)-OVA | 100 | 27.92 |
| CP(AA-MVP)-OVA | 20.33 | 1.08 |

Analysis of SC + PEC mixtures by light microscopy revealed that PEC-1 added to SC suspensions markedly increased the intercellular aggregation which at 0.1 μg/ml OVA was 2%, reaching 40% at 100 μg/ml OVA. Pre-incubation of SC with 10% embryonic calf serum decreased the SC aggregation down to 10%. No cell aggregation was observed in the presence of OVA or its complexes with polyampholytes. Stipulating that protein molecules can be aggregated by short chain polycations whose length is smaller than the circumference of the protein globules (Kabanov and Mustafaev, 1977, 1981), it may be supposed that in our case, when the length of the PEC chains was smaller than the cell size, we dealt with a very similar phenomenon. Cell clusterization occurred as a result of SC interactions with PEC-1 and the dusters formed thereby were stabilize d by relatively short chain macromolecules of PEC whose chain length was much smaller than the cell circumference but sufficient to induce their adherence on the surface of two adjacent cells via the multipoint cooperative interactions between them. After incubation of SC with PEC in the presence of embryonic calf serum PEC-1 added to the mixture appeared to be bound to serum proteins as a result of which the number of free sites of the polycation diminished, eventually resulting in the decreased ability of the complex to interact with SC and to induce their aggregation. In each duster the cells seemed to interact with one another by binding to definite sites on the PEC molecule, such as hydrophobic cetyl radicals of the free sites of PE bound to the membrane lipid bilayer, or fragments containing no cetyl groups but capable of electrostatic interactions with the negatively charged groups situated on the cell membrane surface. As a result of such interactions the negative charge of the SC surface became "neutralized" and it no longer impeded the cell-to-cell contacts. In the case of partial adsorption of PEG-2 the situation was quite different. Partial binding of negatively charged molecules of PEC-2 to cell membranes occurring against electrostatic repulsion might increase the effective negative charge of the cell surface at the expense of polyions "adhering" to it. Quite naturally, this process was unaccompanied by SC aggregation.

One may expect from these data that in living organisms PEC-1 and PEC-2 will behave in a similar way. As a matter of consequence, the ability of artificial polymer-subunit antigens in multipoint cooperative interactions with chemically complementary molecules (blood proteins, intrinsic membrane proteins of immunocompetent cells, etc.) plays a role in their immunogenic activity. This circumstance obviates the need for the development of highly immunogenic artificial antigens.

**5.5. Immunogenicity of multicomponent (chymeric) PEC**

Investigators engaged in the construction of multicomponent artificial vaccinating materials are usually faced with the problem of developing complex antigenic systems for vaccination against several infections at a time. Needless to say, the question "Are PEC mixtures more effective than multicomponent complexes?" comes to the foreground.

In our attempts to perform PEC synthesis we used BSA and BGG as model antigens and a copolymer (CP) of 4-vinyl-Nethylpyridinium bromide with 4-vinyl-N-cetylpyridinium bromide [PVP($R_2$,$R_{I6}$)] as polycationic carrier.

Individual water-soluble complexes of BSA and BGG with CP were obtained as described previously (Kabanov *et al.,* 1977; Mustafaev, 1981). A hypothetical scheme of synthetic PEC is shown in Figure 138. In aqueous solutions each polycation of PC binds two BSA and one BGG molecule. Some hydrophobic radicals of the polycation appear to be bound to the nonpolar regions of the protein, while others interact with one another, thus stabilizing the overall structure. The positively charged fragments of CP form salt bridges with the negatively charged functional groups situated on the surface of the protein globules. Their hydrophobic interactions promote the stability of PEC under physiological conditions. These finding leaves hope that such PEC will not dissociate under conditions of the whole organism.

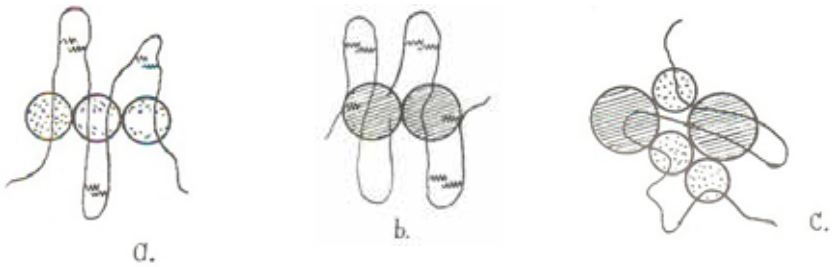



**Figure 138.** A hypothetical structural scheme of the individual complexes PEC-1 (a) and PEC-2 (b), and of a chymeric (PEC-1 – PEC-2) complex (c).

These complexes were further used in immunological studies. To this end we prepared a mechanical mixture of two PEC, (BSA-CP) + (BGG-CP) at a 1:1 ratio. Specially designed experiments revealed that the complexes retained their stability upon mixing which was unaccompanied by their dissociation or by any other structural changes.

The two-component (chymeric) CP-BSA and CP-BGG complexes were obtained as described previously *(*Kabanov *et al.,* 1980). In these complexes one macromolecule of PE was bound to three molecules of BSA and to one molecule of BGG.

To obtain chymeric PEC, equal volumes of aqueous solutions of CP and BSA + BGG were mixed at room temperature (pH 7.5) at the 0.07 g/dl CP + (0.1 g/dl BSA + 0.1 g/dl BGG) ratio. Under these conditions the reaction equilibrium was fully shifted to the left (Figure 140).

In experiments designed to investigate the immunogenic activity of PEC and their mixtures, solutions of pure antigens, individual PEC-1 (CP-BSA), PEC-2 (CP-BGG), their mechanical mixtures and chymeric PEC were intraperitoneally (i/p) injected to mice at a constant (0.5 mg/animal) concentration of the proteins. The AFC levels in the spleen cells of mice immunized with the individual complexes, PEC-1 and PEC-2 and their mixture are shown in Figure 138. It can be seen from these data that immunization of mice with PEC-1 and PEC-2 elicited strong immune responses to both proteins. Interestingly that the immunogenic activity of PE exceeded (almost 10-fold) that observed after immunization of mice with the pure antigens. Immunization of animals with the mechanical PEC-1 + PEC-2 mixture was unaccompanied by any significant changes in the immunogenic activity of these complexes: the AFC levels were high enough, being commensurate in strength with the immune responses to the individual PEC [178].

These results testify to the fact that the immunogenicity of each individual PEC in the composition of mechanical mixtures does not change, i.e., PEC as artificial immunogens function independently, without any antigenic competition.

The situation was quite different when mice were immunized with the two-component chymeric CP + (BSA + BGG) complex. It can be seen from the Figure 139 that in this case the immunogenic activity of BSA and BGG was markedly decreased in comparison with animals immunized with the mechanical mixture of individual PEC. Similar results were obtained during the analysis of AFC titers in the blood sera of mice immunized with the chymeric complexes or individual PEC.

These data suggest that the immunogenicity of PE formed by various protein antigens is higher in mechanical mixtures of individual PEC than in uniform multicomponent complexes. The physicochemical mechanism of this effect consists, apparently, in the ability of free sites of PE within the PEC structure in the cooperative adsorption on the surface of charged cellular membranes, which, in turn, facilitates the antigen interaction with B-lymphocytes. The efficiency of these interactions may increase due to the high epitope density of the antigen within the PE molecule and the more regular distribution of the antigenic determinants, which creates sterically

favourable conditions for the multipoint interactions of the antigen with cell receptors.

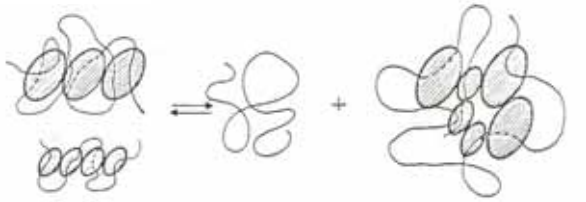


**Figure.139.** The redistribution of BSA and BGG globules between the polycations [PVP($R_2$,$R_{16}$)]

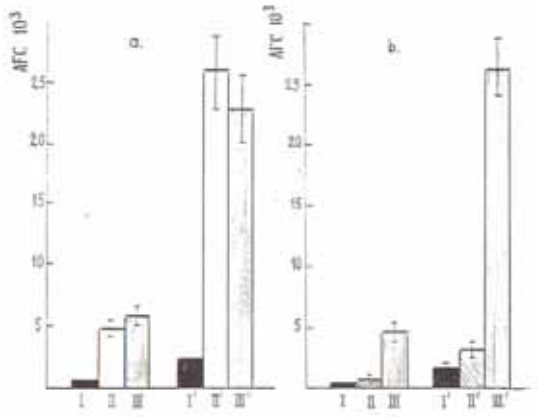



**Figure 140.** AFC accumulation in the spleen cells of mice immunized with BSA (I), BGG (I'), PEC-1 (II), PEC-2 (II') and PEC-1 + PEC-2 (III, III'). (b) AFC accumulation in the spleen cells of mice immunized with (BSA + BGG) (I, I'), PEC-1 – PEC-2 (II, II') and PEC-1 + PEC-2 (III, III').

These results are of paramount importance for they provide a basis for the construction of artificial multicomponent polymer-subunit materials allowing a simultaneous vaccination against several infections and shed lighter on their mechanisms of action.

**The working model of immunogenic PEC**

In our studies we set ourselves a task to construct PEC possessing an immunogenic activity. A model of such immunologically active (or passive) PEC is shown in Figure 141, structure I. The physicochemical criteria that were taken into consideration during the construction of such PEC are as follows.

1. The antigen and the polymeric molecule are linked together by tight bonds that are resistant to physiological conditions and exogenous influences (e.g., multipoint electrostatic and hydrophobic interactions, ion-coordinate and covalent bonds, etc.).

2. PEC have a nonstoichiometric composition. The high epitope density of their antigenic determinants is due to the binding of several antigenic molecules to one macromolecule of PE.

3. PEC have a loop-like organization due to the presence in their composition of flexible free polymeric sites. Excessive ionized groups of PE, both adhering to the protein globule surface and included into the loops, form an extensive hydrophilic area on the PEC surface, which promotes the solubility of the PEC particles.

4. PEC have a rigid rod-like carcass structure which is made up of antigenic molecules stabilized by the polymeric carrier Such a structure promotes the regular distribution of antigenic determinants and allows one to increase the interacting surface.

5. PEC are capable of multipoint cooperative interactions with individual components of

biological systems, such as proteins, immunocompetent cells, etc. This ability is conferred on PEC by free, noninteracting with the antigenic molecules functional groups of PE situated in the loops. The reactivity of PEC within the composition of complex multicomponent system s depends on the surface charge, length and hydrophobic-hydrophilic balance of the se sites. One characteristic feature of highly immunogenic PEC is their ability to form complexes with different components of biosystems.

6. The regular distribution of antigenic determinants creates sterically favourable conditions for multipoint interactions of the antigen with cell receptors. Therefore mixtures of individual PEC are far more effective tools in the construction of polyvalent vaccines than multicomponent (chymeric) complexes.

The structural model of low immunogenic PEC differs from that described above (Figure 141, structure II) and meets the following physico-chemical criteria: tight binding of the antigen to the polymeric carrier, stoichiometric composition of PEC, screening of antigenic molecules from the deleterious effects of environmental factors by the "nonreactive" polymeric envelope surrounding the protein globule, etc.

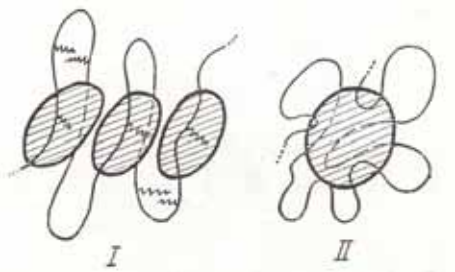



**Figure 141.** The working model of highly (I) and low immunogenic (II) antigen-PE complexes

The physico-chemical mechanism underlying the immunological activity of protein (hapten) complexes with PE consists, in all probability, in the high cooperative adsorptive capacity of free sites of PE that are not involved in the interaction with the protein globules situated on the heterofunctional surface of negatively charged membranes of immunocompetent cells. This process resembles in many features the PE adsorption on the oppositely charged surfaces of colloid particles (so-called flocculation of colloid systems). In this case the polycationic carrier "sticks together" the antigenic substance and the B-lymphocytes triggering immune responses and thus provokes their interaction. The efficiency of such "forced" interactions is high enough for the immune response to be triggered on even in the absence of T - helper lymphocytes or under conditions of their strong deficiency. The T-independence of PEC antigens described earlier in this chapter provides an illustrative example of this phenomenon. Figure 142 is a schematic presentation of a complex antigen attached to the B-cell membrane surface by "sticky" PE sites, which promote its interaction with cell receptors. One may conclude from these data that the sites of PE that are not involved in the binding to the protein antigen will interact via multipoint cooperative complex-forming contacts with the membrane proteins of immunocompetent cells, eventually resulting in the redistribution of these proteins within the lipid bilayer (so-called clusterization of lipids).

This process resembles the events taking place in the cellular membrane in the presence of the original PE. The ability of the polymeric molecule to induce the aggregation of intrinsic proteins of cellular membranes was reported by a group of Japanese investigators who studied the interaction of human red blood cells with polycations [poly(N-vinyl-3-alkylimidazole bromides)] by measuring the fluidity of membrane vesicles in model systems (Tsuchida, 1979) [231]. Similar results were obtained by a Mustafaev and co-workers [230] who studied SRBC interactions with quaternized PVP by using scanning electron microscopy (Figure 143).

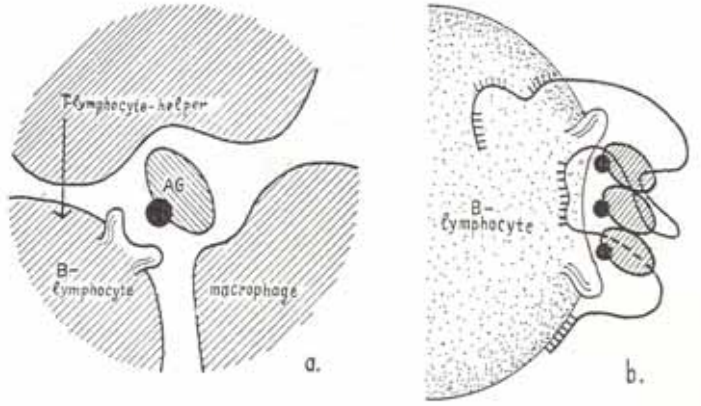



**Figure 142.** The hypothetical schemes of antigen-cellular complexes able to induce T-independent immune responses

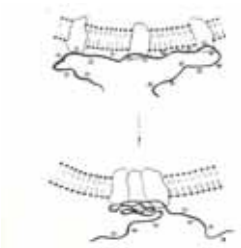


**Figure 143.** Clusterization of cell membrane proteins induced by positively charged PE in the presence of SAS (lipid bilayer).

It seems likely that the mechanism of the PE-induced clusterization of membrane-bound proteins in the presence of lipid molecules resembles, in some features, the mechanism of self-assembly of PEC formed from linear PE and globular proteins in diluted aqueous media (Kabanov and Mustafaev, 1977, 1981). This mechanism was confirmed by the results of model studies carried out in the past decade.

Our studies showed that the binding of linear polycations to globular proteins is practically insensitive to the addition of negatively charged molecules of surfactants, e.g., SDS. We succeeded in demonstrating that within a rather broad range of [SAS]/[BSA] molar ratios in the PVP($R_2$) - SAS-BSA system the mode of binding is always the same. The reaction occurs via the "all or nothing" mechanism and consists in the self-assembly of asymmetrical aggregates from ternary PEC particles. As for SAS, they exclusively affect the composition and stability of the complexes being formed [9,9a].

Specially designed experiments employing the use of electron microscopic and luminescent methods were aimed at the elucidation of the ability of intrinsic membrane proteins of major immunocompetent cells to form clusters with PE purified from protein antigens as well as at the clarification of their role in the immunomodulating activity of synthetic PE. However, all these studies were conducted with cells separated from serum proteins, i.e., in serum-free media; therefore the conclusions made the re in should not be generalized. Valuable information can be derived from physico-chemical analyses of cell-PEC mixtures. Serum proteins added to such mixtures induced multipoint interactions between the cell membrane surface and PEC molecules resulting in the reciprocal aggregation of antigen-cell particles.