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Biochemical Applications of Poly(Ethylene Glycol)

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The literature data on the use of poly(ethylene glycol) (PEG) in biochemistry and molecular biology are analysed from the standpoint of the physical chemistry of polymers. The exclusion properties of PEG, used as a factor influencing the rate of enzymatic reactions, the intra- and intermolecular transitions in polymers, and biopolymer precipitation and crystallisation processes are examined. The suitability of three-component PEG-dextran-water mixtures for the purification and separation of mixtures of biopolymers and for analytical purposes as well as the possibility of using functional derivatives of PEG in immunological research and for the immobilisation of enzymes are discussed. A special section is devoted to the synthesis of PEG derivatives containing different functional groups at the ends of the polymer chain. The surface-active properties of PEG are examined.
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I. INTRODUCTION

The interest in poly(ethylene glycol) (PEG) has been recently stimulated by its successful application in biochemistry and molecular biology, where this polymer is widely used in different kinds of research. Analysis of literature data shows that the experience accumulated by biochemists in the use of PEG (mainly of its aqueous solutions) for the purification and separation of natural products and for analytical purposes can be extended and applied successfully in the solution of certain problems of the physical chemistry of high-molecular-weight compounds. On the other hand, these studies necessitate a more detailed investigation of the chemical and physicochemical properties of PEG and especially of reactions involving its end groups.

Furthermore, there have been a number of studies of the effect of PEG on certain processes in living systems. In its turn, this faces chemists with the problem of the study of the molecular mechanisms of such effects and leads to new prospects for the investigation of the physicochemical properties of PEG and its possible applications.

The aim of this review is to acquaint chemists, mainly specialists in the field of high-molecular-compounds, with experimental data on the applications of PEG in biochemical research and an attempt is made to interpret these results from the standpoint of the physical chemistry of polymers.

II. EXCLUSION PROPERTIES OF POLY(ETHYLENE GLYCOL)

The widest applications of PEG in biochemistry are associated with its use as an exclusion agent. The term "exclusion" was first introduced in biochemical literature and is in essence a synonym of the concept of the "incompatibility of polymers" familiar in the physical chemistry of polymer solutions.

The exclusion principle can be explained by considering the question of the influence of the presence of polymers on the properties of low- and high-molecular-weight substances in solution. In polymer solutions the mobility of small ions and molecules is determined by the "microscopic" viscosity of the system, which becomes appreciable in the range of polymer concentrations where the macromolecular coils appreciably interpenetrate one another (critical concentration). At lower concentrations the microscopic viscosity of the system is small and the diffusion coefficients of small molecules therefore change very little. On the other hand, if another high-molecular-weight compound is introduced into a solution containing a linear polymer, then an apparent increase of the true concentration of the components in relation to the case where the substances are present in solution in a pure state is observed. This effect is usually interpreted as the mutual exclusion of the macromolecules from part of the solution. The hypothesis of exclusion is based on the observation that the change in chemical potentials is a function of the size of the excluded molecules[1].

Studies on the influence of polymers on biochemical processes can be divided into three groups.

1. The study of enzymatic reactions in polymer solutions, i.e. in systems simulating the environment of intracellular enzymes.
2. The study of intra- and inter-molecular transitions in polymers in the presence of exclusion agents.
3. The use of polymers for the precipitation and crystallisation of biopolymers.

The point is that in vitro biochemical processes are in most cases investigated in aqueous buffer solutions. However, the same reactions in vivo take place in the presence of high concentrations of high-molecular-weight substances—proteins, nucleic acids, and carbohydrates, which influence various chemical equilibria.

The first step in the study of enzymatic reactions under conditions approaching those in nature involves enzymatic reactions in the presence of neutral linear polymers which do not interact specifically with the components of the enzymatic reaction. When a polymer is introduced into the system, the chemical activity of enzymes, substrates, and inhibitors increases. Consider a one-substrate reaction described by the equation

$$E+S\rightleftharpoons ES\rightarrow E+P \quad ,$$

where E is the enzyme, S the substrate, ES the enzyme–substrate complex, and P the reaction product. The dissociation constant of the enzyme–substrate complex can be formulated as follows:

$$K = \frac{\gamma_E \gamma_S}{\gamma_{ES}} \cdot \frac{c_E c_S}{c_{ES}}.$$

We shall consider how the size of the reaction components affects the ratio of the activity coefficients†. If the substrate has a low molecular weight, one can assume that the size of the enzyme molecule and of the enzyme–substrate complex and hence also their activity coefficients are similar. The activity coefficient ratio will therefore depend on γS; since the latter quantity apparently differs little from unity, the entire activity coefficient ratio will be close to unity. Thus, in the conversion of lactate into pyruvate catalysed by lactate dehydrogenase,

$$\text{lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+,$$

$\gamma_S = 0.76$ for pyruvate and lactate in 23% dextran gel, while its value for NAD^+ is 0.62.[1]

On the other hand, if the substrate is a high-molecular-weight compound, whose molecular size significantly exceeds the molecular size of the enzyme, the activity coefficient ratio will be determined by the activity coefficient of the enzyme γ_E, which is as a rule appreciably greater than unity; for example, $\gamma_E = 4$ for the hyaluronic acid–hyaluronate lyase–dextran system and the Michaelis constant K_M should increase approximately by a factor of four. The same order of magnitude of the changes in K_M is in fact observed for enzymatic systems under experimental conditions.

In addition to increasing the chemical potentials, the presence of polymers in solutions can cause a decrease of the diffusion coefficients of other system components, manifesting the so-called sieve effect[1]. This leads to a decrease of the frequency of collisions between the reactants and to a decrease of the maximum rate of reaction v_{max}. However, this effect should not influence the equilibrium constants for the reactions.

We may note that the view that the action of polymers on enzymatic reactions involves energy exchange between the polymeric medium and the enzyme–substrate complex has been expressed in a number of studies#.

The second group of studies is associated with the investigation of intra and inter-macromolecular processes in biopolymers. If the macromolecules can exist in solution in different conformational states characterised by different molecular volumes, then the presence of another polymer in the system should lead to different exclusions of different forms and in the presence of the added polymer the conformation having the smallest volume should be stabilised. The study of the conformational transitions of three polynucleotides from an ordered to a disordered conformation in the presence of dextran and PEG showed[2] that the increase of the stability of the ordered conformation depends on the concentration of the added polymers, PEG showing a higher efficiency in exclusion than dextran. The increase of the stability of the ordered conformation was inferred in the above study[2] from the change in the hypochromic effect as a function of temperature. Two families of melting curves for the poly(deoxyadenylic–thymidylic acid) helix, measured in the presence of PEG of different molecular weight and different dextran fractions, were obtained (Fig.1). The curves in Fig.1*a* show that the change in the melting temperature of the helix depends significantly on the molecular weight of the PEG for the low-molecular-weight fractions, while the dependence is slight for high-molecular-weight specimens (molecular weights of 4000 and 6000). In the case of dextran all the specimens had a high molecular weight, so that the melting temperature of the polynucleotide helix were the same in all cases.

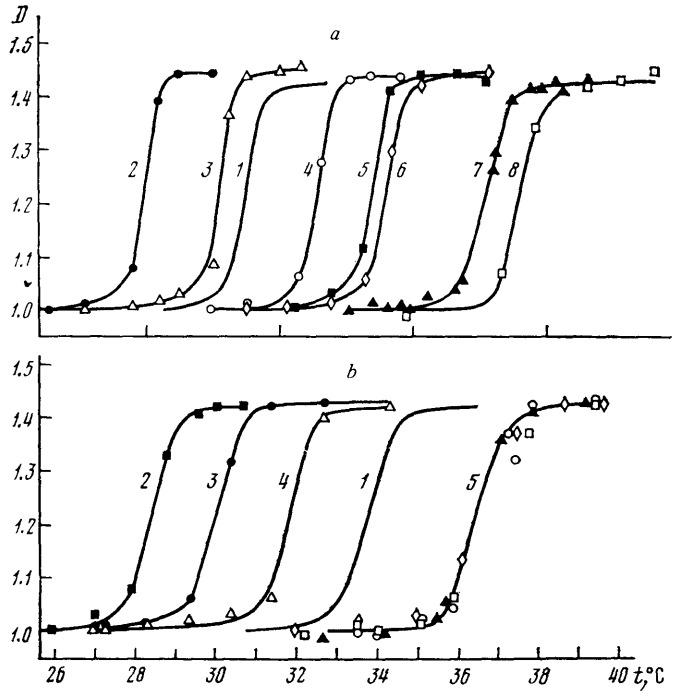


Figure 1. The influence of the molecular weights of PEG (*a*) and dextran (*b*) on the melting of the poly[d(A – T)·d(A – T)] helix in a saline tris buffer[2]; D = optical density at 260 nm: *a*) PEG concentration 20.6 g/100 ml of buffer [1) without addition of polymer; 2) monomer; 3) PEG-200; 4) PEG-400; 5) PEG-600; 6) PEG-1500; 7) PEG-4000; 8) PEG-6000]; *b*) dextran concentration 19.3–19.9 g/100 ml of buffer [1) without addition of polymers; 2) sucrose; 3) glucose; 4) raffinose; 5) dextran-10, dextran-40, dextran-80, dextran-500].

†The activity coefficients γ are found from the relation $K_{av} = 1/\gamma_E$, where K_{av} is the partition coefficient of the given substance between the polymer gel at a known concentration and a buffer.

#S.Damjanovich and B.Somogyi, "Symposia Biologica Hungarica. New Trends in the Description of the General Mechanism and Regulation of Enzymes", Academiai Kiado, Budapest, 1978, pp.159–184.

A good illustration of the exclusion effect is provided by the conversion of a two-stranded DNA molecule into a compact form in an aqueous salt solution after the addition of PEG.[3] This process can be regarded as a model of the structural transitions of DNA in the cell occurring under the influence of of polymers of biological origin.

The idea of using linear water-soluble polymers to act upon other important equilibrium processes in a living organism, such as, for example, the enzyme association–dissociation processes, proved to be very attractive in this connection. This possibility was first pointed out by Laurent[1]. It was realised experimentally in a study[4] where a system containing the enzyme phosphorylase B, which is capable of association, was treated with PEG having a molecular weight of 40000 at a concentration of 4 g/100 ml. The enzyme association process was followed by the sedimentation method in an ultracentrifuge. The enzyme concentration in the system without PEG is selected in such a way that the contents of the dimer and tetramer are 55 and 45%. In the presence of PEG the dimer ⇌ tetramer equilibrium is displaced towards the formation of the tetramer, whose fraction increases to 75%. One should note that in the above study[2] on the compaction of polynucleotides use was made of PEG solutions at concentrations higher by an order of magnitude than in the investigation of Kurganov et al.[4] In the latter case the method of observation precludes the use of such high polymer concentrations in view of the marked increase of the viscosity of the system; this can probably explain the relatively small influence of PEG on the association of the dimeric form of phosphorylase B. Furthermore, the use of higher PEG concentration becomes impossible because the enzyme precipitates at a PEG concentration exceeding 5 g/100 ml.

Yet another striking example of the important role of the polymeric medium in biochemical processes is provided by the observation of the interaction between unlike proteins in the presence of PEG. The following pair of enzymes were investigated: malate dehydrogenase–glutamate dehydrogenase§ and malate dehydrogenase–citrate synthase. It is important to note that the interaction of these enzymes, which are involved in the Krebs cycle, could not be detected in buffer systems in vitro.

Finally, the third region of application of the exclusion effect, which is most important in practice, is associated with the ability of linear polymers to induce the phase separation of the system, i.e. the precipitation of other macromolecular compounds. In this case an appreciable increase of the activity coefficient of the added polymer leads to attainment of the solubility limit by the latter. Together with dextran, PEG is widely used to precipitate proteins[5].

In comparing the abilities of PEG and dextran to induce the precipitation of proteins, one may note that PEG is three times more effective than dextran, in agreement with data obtained in the study of the influence of these polymers on the kinetics of enzymatic reactions[1].

A study has been published recently[6] illustrating the ability of PEG to induce the crystallisation of proteins from aqueous solutions. It has been shown for the crystallisation of 22 proteins that PEG is the most universal reagent compared with those employed previously. The study of the morphology of protein crystals isolated from PEG solutions showed that the structure of the crystals is the same as that of the preparations obtained by the usual methods.

Inspection of the literature data on the exclusion processes occurring in the presence of different water-soluble polymers shows that the most effective exclusion agents are PEG,[1,7–11] dextran[1], and hyaluronic acid[12–14]; polyvinylpyrrolidinone and poly(vinyl alcohol) are less effective[15].

The excluded volumes in systems containing different polymers with the same "partner" can serve as the most natural criterion for the comparison and estimation of the exclusion properties of polymers; however, such data are not available in the literature. Measurement of the excluded volumes is difficult[16]. Ogston¶ and Laurent[17] succeeded in greatly simplifying this problem by considering the simplest model of the interaction of a linear polymer, which can be represented by a long thin rod, with globular particles which are assumed to be spherical (Fig.2). The greatest exclusion effect, expressed as the excluded volume for S, referred to unit mass of R, observed for asymmetric R. The centre of the spherical particle S cannot approach R to a distance less than r_S. The excluded volume for the centre of S is equal to the volume of the cylinder bounded by dashed lines whose radius is equal to the sum of the radii $r_S + r_r$ (r_r = the radius of the rod). If the end group effect can be neglected, then the volume excluded for the sphere is $\pi l_r (r_S + r_r)^2$, where l_r is the length of the rod. If d is the density of the rod, then its mass is $\pi l_r r_r^2 d$ and the excluded volume v_{excl}, referred to unit mass of R, is

$$v_{excl} = \frac{\pi l_r (r_S + r_r)^2}{\pi l_r r_r^2 d} = \frac{1}{d}\left(\frac{r_S}{r_r} + 1\right)^2.$$

The degree of exclusion increases with increasing radius of S and with decreasing radius of R or with increasing asymmetry of the polymer.

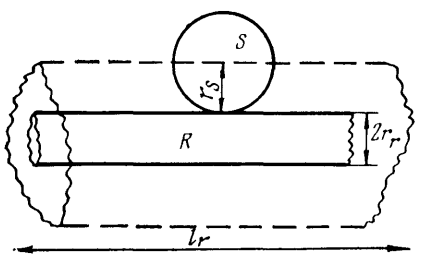


Figure 2. Scheme explaining the phenomenon of the exclusion of a spherical particle by a rodlike particle.

If a series of rods are distributed at random in space, the excluded volumes for individual rods should overlap. The expression for K_{av}, the overall volume accessible to spheres with radius r_S in the space containing randomly distributed long rods with radius r_r, is

$$K_{av} = \exp[-\pi L (r_S + r_r)^2],$$

where L is the sum of the lengths of all the rods per unit volume. We may note that the concentration of the rods (i.e. L) and the square of the radius of the sphere (r_S^2) are the only parameters which determine the accessible volume for $r_r \ll r_S$.

The fraction of the volume accessible for the given substance (K_{av}) is an extremely important parameter for the determination of the exclusion properties of the system. It is related to the excluded volume by the expression

$$v_{excl} = (1 - K_{av})/c_R,$$

where c_R is the concentration of the rods R expressed in terms of mass referred to the volume.

§L.A.Fahien, K.Kmiotek, and L.Smith, Arch.Biochem. Biophys., 192, 33 (1979).

¶Quoted by Laurent[17].

Despite its simplicity, this theory proved to be entirely applicable to the description of the exclusion properties of certain polysaccharides, in particular dextran and hyaluronic acid[14]. It permits the calculation of K_{av} by Eqn.(5) from the values of r_S, r_r, and L. Comparison of these quantities with the experimental values obtained from gel-filtration data shows an excellent agreement. The linear hyaluronic acid is 4–5 times more effective than the branched dextran. Special experiments showed[8] that 5–10% solutions of dextran have the same exclusion properties as dextran gels at the same concentration. The values of K_{av} and hence the activity coefficients of the substances excluded by the polymer gel can therefore be used subsequently to calculate the activity coefficients of the substances present in solutions of the corresponding polymers.

The excluded volumes in three-component systems containing PEG, a globular polymer, and water, have been found by Edmond and Ogston[18] by measuring the thermodynamic parameters in systems with phase separation.

Adopting the Ogston–Laurent hypothesis as a basis for the description of exclusion effects, one should also bear in mind that polymers influence the structure of water[19–22]. It follows from NMR data for aqueous solutions of PEG that each oxygen atom in the polymer chain binds up to 16 water molecules[23]. Thus PEG has a structure-forming influence on several molecular layers of water. It may be that the structure-forming effect of PEG on water is in fact one of the causes of its appreciable exclusion properties.

One should note that the exclusion principle, which obtains in living organisms and is widely used in molecular biology, can be applied successfully also in the chemistry of high-molecular-weight compounds in order to carry out tasks such as the increase of the specificity of reactions between macromolecular components, the crystallisation of water-soluble polymers, etc.

III. THE USE OF THREE-COMPONENT POLY(ETHYLENE GLYCOL)–DEXTRAN–WATER SYSTEMS FOR PREPARATIVE AND ANALYTICAL PURPOSES

The same exclusion principle, namely the mutual displacement of polymers from part of the solution, is also responsible for phase separation in systems consisting of two polymers in a common solvent. When aqueous solutions of dextran and PEG at concentrations above the critical value are mixed, a two-phase system is formed in which PEG is contained in the upper phase and dextran in the lower phase. Such systems, which can be "buffered" and made isoosmotic, are widely used for the separation of biomacromolecules, cell particles, viruses, membranes, chloroplasts, etc. by partitioning the relative substances between different phases[24]. Together with the dextran–PEG pair, other pairs incorporating modified derivatives of these polymers are also used, for example, dextran–methylcellulose, dextran sulphate–dextran, dextran sulphate–carboxymethyldextran, etc.

The specific features of three-component systems containing two polymeric components are such that phase separation occurs even for polymers with very similar chemical structures, present in solution at low concentrations. The main application of two-phase systems consist in the separation of substances by partitioning between two polymeric phases. This method of separation is more effective than other procedures—electrophoresis and sedimentation[24].

The most remarkable property of the PEG–dextran system, containing non-ionogenic polymers, is the influence of electrolytes on the partition of substances between phases[25]. The fact that a given substance can be transferred from one phase to another simply by adding, for example, NaCl in excess relative to sodium phosphate or by substituting Li^+ ions by Na^+ ions is of interest from both theoretical and practical points of view. It has been noted[26] that negatively charged biomacromolecules have higher partition coefficients in two-phase systems containing sodium sulphate than in those containing sodium chloride; the opposite behaviour has been observed for positively charged compounds. Plots of the partition coefficients K of proteins in phases containing Na_2SO_4 and NaCl against pH consist of two curves intersecting close to the isoelectric point[24]. Such crossed curves are illustrated in Fig.3 for bovine serum albumin[27]. Using two-phase systems at different pH, Sasakawa and Walter[27] obtained the isoelectric points also for a number of other proteins and investigated the dependence of the isoelectric points of haemoproteins on the molecular weight. Blomquist[28] obtained the isoelectric points for the enolase isoenzymes. There is no doubt that this method can be used successfully also for the determination of the isoelectric points of synthetic polyelectrolytes.

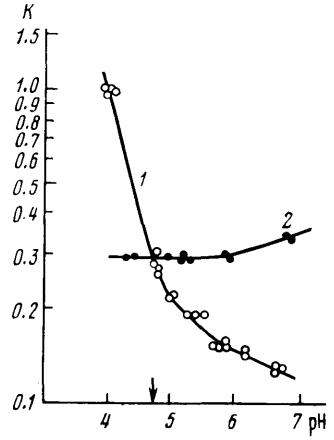


Figure 3. The cross-distribution curves for bovine serum albumin; pH dependence of the partition coefficient K in a two-phase system containing NaCl (curve *1*) and in a system containing Na_2SO_4 (curve *2*).

It is believed[24] that there is a non-uniform distribution of ions between the phases of the PEG–dextran–water system, which leads to a potential difference between the phases defined by the relation

$$U_2 - U_1 = \frac{RT}{(z^+ + z^-)F} \ln \frac{KB^{z^-}}{KA^{z^+}}, \qquad (1)$$

where z^+ and z^- are the numbers of positive and negative charges arising on partitioning the salt A_z-B_z+, F is the Faraday, and K_{Bz^-} and K_{Az^+} are the partition coefficients of the anion and cation respectively.

This potential difference influences in its turn the distribution of the polyelectrolyte or charged species, such as those of proteins and nucleic acids. As a result of the large number of charges on the biomacromolecules, the behaviour of such species in the partitioning process depends strongly on the interfacial potential even if the latter is relatively small.

The problem arises of the cause of the non-uniform distribution of different inorganic electrolytes between the phases. It is believed[25] that cations exhibit a greater affinity for PEG than for dextran, the affinity decreasing in the sequence $Li^+ > Na^+ > K^+$. At the same time, an increase of the affinity of the anions for PEG has been noted in the series of halides with decrease of their polarisability in the sequence $I^- > Br^- > Cl^-$. On the other hand, it has been found[25] that SO_4^{2-} and HPO_4^{2-} have a greater affinity for dextran. These phenomena are due to differences in the tendencies towards the formation of ion pairs in the two phases.

The introduction of charged groups at the ends of the PEG molecule has an even greater influence on the interfacial potential. Typical examples of charged PEG are bistrimethylamino-PEG and PEG-bissulphonate[29]:

$$(CH_3)_3\overset{+}{N}CH_2CH_2OCH_2CH_2O \ldots OCH_2CH_2\overset{+}{N}(CH_3)_3$$
$$^-O_3SCH_2CH_2OCH_2CH_2O \ldots OCH_2CH_2SO_3^- .$$

When these polymers are introduced into the PEG–dextran two-phase system, they move to the PEG-containing phase, as a result of which the system acquires the properties of a liquid ion exchanger.

The decisive factor in the distribution of proteins between the phases is their charge. On the other hand, the required distribution of substances could be attained either by introducing charged groups into the dextran molecule, using, for example, DEAE- or CM-dextrans, or by adding exogenous polyelectrolytes to the system. However, in this case the components of a polyelectrolyte two-phase system have such a high charge density in their molecules that, in conformity with Eqn.(7), the potential difference between the phases diminishes. Furthermore, dextran derivatives frequently migrate to the PEG-containing phase. Finally, the formation of polymeric complexes between polyelectrolyte molecules and cell particles, which are difficult to dissociate, has been noted[24]. Thus the main advantage of charged PEG is that the charges are located solely at the ends of the polymer and the macromolecules retain their affinity for the PEG-containing phase[24].

In his study of the distribution of proteins in two-phase systems, Albertson (quoted by Blomquist[28]) proposed a method which makes it possible to determine the surface charge of proteins. He derived the following equation which makes it possible to relate the partition coefficient of the protein K to the overall molecular charge z and the interfacial potential ψ:

$$\ln K = \ln K^0 + zF\psi/RT,$$

where K^0 is the partition coefficient of the protein having an overall charge of zero.

The use of two-phase systems also made it possible to discover a new method of estimating the hydrophobic properties of macromolecules and small molecules[30]. For this purpose, the end groups of PEG were modified with hydrophobic ligands, in particular, they were esterified by aliphatic acid residues. The PEG modified in this way moves to the same phase as the unsubstituted polymer and makes it less polar. By studying the distribution of a large number of compounds in the same system containing 80–95% of water, it is possible to compare their hydrophobic properties. Using this method, it is natural to adopt as a measure of the hydrophobic properties of the given substance the difference between the free energies of the transfer of 1 mole of the substance from the upper phase of the system containing unsubstituted PEG to the upper phase of another system containing the hydrophobic ligand bound to the polymer:

$$\Delta G'' - \Delta G' = -2.3\, RT\, \Delta \lg K, \qquad (2)$$

where $\Delta G''$ is the free energy of the transfer of 1 mole of the substance from one phase to another in a system containing a hydrophobic polymeric ligand and $\Delta G'$ is the corresponding quantity for a standard two-phase system containing unsubstituted PEG.[30] The quantity $\Delta \lg K$ in Eqn.(2) is defined as follows:

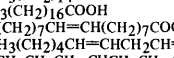
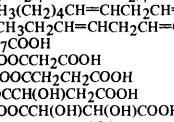
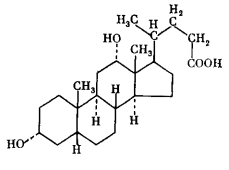
$$\Delta \lg K = \lg K_0'' - \lg K_0',$$

where K_0'' and K_0' are the distribution constants of the substance in the system containing the hydrophobic ligand and in the standard system.

This method for the determination of the hydrophobic properties of substances makes it possible to eliminate effects due to the electrostatic interactions between the molecules of the polymeric ligand and the protein. Another advantage of the method is that the hydrophobic interactions can be investigated in water, in the absence of organic solvents.

The use of hydrophobic ligands bound covalently to PEG constitutes one of the examples of the introduction of affinity ligands into PEG in order to enhance the specificity of the separation of natural products. The same principle has been used for the specific extraction of a number of proteins[31]. For example, a dinitrophenylated derivative of PEG has been used to isolate the S-23 myeloma protein from a mixture[32]. In order to purify steroid-binding proteins, such as plasma transport proteins, PEG containing oestradiol residues as end groups was synthesised as a ligand with a high affinity for the protein[33].

Table 1. Poly(ethylene glycol) esters of various acids.

Acid used to esterify PEG	No. of carbon atoms in acid residue	Degree of substitution, %
Acetic CH_3COOH	2	17
Butyric $CH_3(CH_2)_2COOH$	4	12
Caproic $CH_3(CH_2)_4COOH$	6	9
Caprylic $CH_3(CH_2)_6COOH$	8	12
Capric $CH_3(CH_2)_8COOH$	10	9
Lauric $CH_3(CH_2)_{10}COOH$	12	7
Myristic $CH_3(CH_2)_{12}COOH$	14	8
Palmitic $CH_3(CH_2)_{14}COOH$	16	9
Stearic $CH_3(CH_2)_{16}COOH$	18	11
Oleic $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	18	11
Linoleic $CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	18	6
Linolenic $CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2{=}CH{=}CH(CH_2)_7COOH$	18	6,5
Malonic $HOOCCH_2COOH$	3	15
Succinic $HOOCCH_2CH_2COOH$	4	16
Malic $HOOCCH(OH)CH_2COOH$	4	11
Tartaric $HOOCCH(OH)CH(OH)COOH$	4	6
Fumaric $HOOCCH{=}CHCOOH$	4	4
Benzoic 	7	4
Nicotinic 	6	4
Trichloroacetic CCl_3COOH	2	1
Bromoacetic $BrCH_2COOH$	2	36
Iodoacetic ICH_2COOH	2	5
Deoxycholic 	24	8

Affinity partition can also be used as an analytical method for the measurement of the binding constants and of the number of binding centres in proteins in relation to different ligands[34]. For this purpose, a number of PEG esters of carboxylic acids, both monobasic and dibasic with different numbers of carbon atoms as well as acids containing a double bond, hydroxy-groups, etc., have been synthesised (see Table 1).

Serum albumin (SA) is known to have a high affinity for aliphatic acids. This explains the significant influence of the addition of PEG esters of higher aliphatic acids to the two-phase system on the distribution of SA. In the standard system SA is displaced predominantly to the dextran-containing phase. In the presence of PEG esters, which migrate to the same phase as PEG, the aliphatic acid residues are absorbed on the SA surface.

SA is known to have seven centres which strongly adsorb long-chain aliphatic acids ($K_{ass} = 10^6-10^8$ litre mol^{-1}) and twenty centres with a much lower affinity ($K_{ass} = 10^2-10^3$ litre mol^{-1}).[34] If only strong binding is considered, it is seen that the protein can bind seven molecules of PEG esters. The resulting complex exhibits an affinity for the upper, PEG-containing phase.

An equation has been obtained[35] establishing a relation between the partition coefficient and the chemical structure of the partitioned substance; according to this equation, the introduction of a new chemical group of the given type into the molecule of the complex alters lg K by a constant amount[35]:

$$\lg K = \lg K^* + n\alpha,$$

where K is the distribution constant of the SA–PEG ester complex, K^* the distribution constant of free SA, n the number of PEG ester molecules in the complex, and α the increment in lg$K$ following the introduction of each new ester molecule.

It has been shown[34] that the equation holds for SA–PEG ester complexes. Using different PEG esters, the increment α was determined and the values of n were calculated. In the calculations no account was taken of the decrease of the accessible surface of the protein with increasing number of bound ligand molecules. If this effect is not very great, the distribution in two-phase systems containing PEG esters can serve as a convenient method for the determination of the number of binding centres in the protein molecule. The binding curves (the dependence of lg$K$ on the ester concentration) can be used to calculate the binding constants.

This method for the investigation of the binding of proteins to ligands is particularly convenient when it is necessary to work with small amounts of protein.

In such cases radioactive labels are used to detect proteins in solution. In this respect, the method for the investigation of binding using two-phase systems has advantages over the method of equilibrium dialysis. Another possibility of using "affinity partition" involves the determination of the type of binding centres present in biopolymers, membranes, and cells.

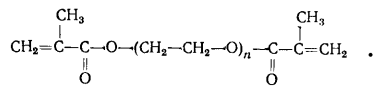
Thus the method based on the distribution of substances in two-phase systems permits not only the purification and separation of biomacromolecules but also yields much information about their various properties, about dimerisation and aggregation, about denaturation under certain conditions, and about the change in the charge of the proteins as a function of pH. The method can also be employed to determine the surface charge of the molecules, their isoelectric points, relative hydrophobic properties, the extent of binding to different ligands, etc.

IV. BIOCHEMICAL ASPECTS OF THE USE OF MONO- AND BI-FUNCTIONAL DERIVATIVES OF POLY(ETHYLENE GLYCOL)

In the description of the properties of two-phase systems the use of disubstituted PEG was already mentioned above[29, 32,33,67], although the need for specifically bifunctional derivatives was not considered.

One of the promising applications of disubstituted derivatives of PEG involves their use as cross-linking agents in the immobilisation of enzymes[36].

One study has already been made on the use of the bifunctionality of PEG in the immobilisation of enzymes[37]. In this study the PEG end groups were esterified by methacrylic acid, which yielded PEG dimethacrylate:



When PEG preparations with different molecular weights were used, polymers were obtained containing at the ends photosensitive functional groups located at the required distance from one another. Next, a mixture, containing the given polymer, enzyme, or microbial cells, was prepared and irradiated for several minutes with ultraviolet light. This yielded matrices cross-linked via their ends and containing the biological materials enclosed in them. The enzyme yeast invertase was immobilised in this way. The photosensitive polymers based on PEG have the following advantages: they form mechanically strong networks, are chemically inert, and are sufficiently permeable to the substrate and the products of the enzymatic reaction. This example by no means exhausts the possibilities of the use of PEG as a cross-linking agent.

In connection with the development of research into the synthesis of medicinal polymeric compounds (MPS), we think it would be extremely useful to synthesise and investigate the properties of such compounds based on PEG. The problem of the determination of the correlation between the structure of the MPS and its physiological activity, which is fundamental, can be solved most rigorously when only a controlled number of pharmacologically active fragments is introduced into the polymer chain. This approach can be used via the chemical modification of the end groups in macromolecules whose functionality is known beforehand.

PEG having hydroxy-groups at the ends of the chain meets these requirements fully.

On the other hand, a necessary requirement which must be met by polymeric carriers, is the immunostimulating activity of the polymer itself. This requirement is also satisfied by PEG, which exhibits the properties of an immunostimulant†.

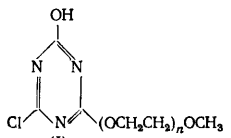
The literature data available at present on the MPS based on PEG involve only one field of application—immunology. The possibility of employing mono- and bi-functional PEG derivatives as antigens for the study of the mechanism of the antigen–antibody interaction has been studied[38]. The interaction of antibodies isolated from immunised animals, with mono- and di-substituted derivatives of 2,4-dinitrophenyl-PEG has been investigated by methods involving the quenching of fluorescence[39] and light scattering[40]. The experimental data obtained in these investigations[38–40] made it possible

†I.N.Topchieva, N.Yu.Alekseeva, V.P.Evdakov, R.V.Petrov, I.V.Savinova, E.D.Filatova, L.I.Khaustova, R.M.Khaitov, and M.B.Temchenko, "Tezisy Doklada na IV Vsesoyuznom Nauchnom Simpoziume "Sinteticheskie Polimernye Materialy Meditsinskogo Naznacheniya"" (Abstracts of Reports at the IVth All-Union Scientific Symposium "Synthetic Polymeric Materials for Medical Purposes"), Dzerzhinsk, 1979, p.5.

to observe structural differences between the binding centres of immunoglobulins, to estimate the distances between the binding centres for two types [class IgG and IgG(T)] of antibodies, and to assess the differences between their conformations in solution.

An important stage in the study of PEG derivatives containing antigenic determinants is the investigation of their activity in vivo. The 2,4,6-trinitrophenylethyl ether of PEG with a molecular weight of 40000, which exhibited a high antigenic activity, was synthesised for this purpose†.

Studies on the chemical modification of proteins with PEG derivatives, which alters the immunological properties of the former, are of great interest[41–43]. The reagent was PEG with a molecular weight of 2000, in which one end group contained the 2-chloro-4-hydroxytriazine moiety, capable of interacting with the amino-groups of the protein, while the other was a hydroxy-group converted into the methoxy-derivative:



(I)

When catalase is treated with this reagent, it is possible to substitute up to 60% of the amino-groups while retaining the enzymatic activity of the protein[41]. A higher degree of substitution of the amino-groups by the reagent reduces the enzymatic activity of catalase. A catalase preparation with a 75% degree of substitution (PEG-catalase) proved to be inactive in relation to the antiserum for native catalase, which was established by gel diffusion and complement fixation methods. The introduction of PEG-catalase into the blood of mice did not lead to the production of antibodies against both PEG-catalase and native catalase. Thus chemical modification of catalase by the PEG reagent yielded a modified enzyme without immunogenic properties. These studies constitute the first step on the way to the solution of the problem of reducing the immunogenic properties of proteins, which will make it possible to extend significantly the therapeutic applications of exogenic enzymes.

A patented method involving the reductive alkylation of the NH_2 groups of enzymes using an aldehyde-derivative of methoxy-substituted PEG and sodium tetrahydroborate was devised in a continuation of the above investigation[42]. The method makes it possible to obtain enzyme preparations with a higher activity and stability compared with those obtained previously.

Reagent (I) has been used[43] for the chemical modification of antigen E, the principal protein allergen of ambrosia pollen. The aim of this modification was to obtain an antigen derivative with a reduced allergenic activity but retaining the immunogenic properties of the native molecule. According to the generally accepted concept, the allergenic activity consists in the liberation in the cell of chemical mediators of allergic symptoms in the interaction of polyvalent antigens with immunoglobulins of class IgE. Chemical modification of antigens, leading to a decrease of the number of accessible antigenic determinants or to a decrease of the affinity for specific antibodies, should reduce their allergenic activity. By controlling the degree and type of chemical modification, it is possible to obtain mixtures of derivatives partly (but not fully) retaining the immunogenic properties of the native protein.

In the interaction of the PEG-reagent with antigen E, containing 18 lysine amino-groups, on average 11 lysine residues reacted. The study of the immunological properties of the PEG derivative of antigen E showed[43] that the latter stimulates the production of specific antibodies, although it is a weaker immunogen than the native protein. At the same time, the PEG-antigen shows a markedly reduced allergenic activity compared with the native antigen. The properties of several derivatives of antigen E, modified via their amino-groups, namely PEG and dextran derivatives, as well as two other derivatives obtained as a result of the introduction of low-molecular-weight groups (*N*-carboxymethyl and butyrimidato-) into the molecule, have been compared[43] (Table 2). Evidently the PEG derivative undergoes a greater decrease of allergenic activity compared with the dextran derivatives, despite the fact that the molecular weight of dextran significantly exceeds that of the fragment of the polyoxyethylene chain. It is of interest to note that both these derivatives have the same molecular volumes, which follows[43] from the identity of their elution volumes in gel chromatography on Sepharose 6B.

Table 2. The properties of derivatives of antigen E modified at the lysine amino-group[43].

Derivative of antigen E	Molecular weight	No. of modified lysine residues	Molecular weight of introduced group	Overall charge of modified antigen	Relative allergenic activity
Native protein	38 000	0	0	−9	1
PEG derivative	53 000	7	2 100	−16	0.024
N-Carboxymethyl derivative	39 000	16	58	−31	0.56
Butyrimidato-derivative	39 000	16	72	−9	1
Dextran derivative	140 000	4	25 000	−9	0.15

This finding can probably be explained on the basis of the difference between the degree of compactness of the PEG and dextran macromolecules in aqueous solutions. The more compact dextran molecules are less effective as regards the steric blocking of the antigenic determinants compared with the less compact PEG groups. We are probably dealing in this instance with exclusion effects generated by different polymers, which are now bound covalently to the molecules of the antigen protein, in relation to the antibody proteins. Examination of the mechanism of the action of PEG-containing proteins from this point of view makes it clear why the modification of the protein by the PEG reagent does not affect its interaction with low-molecular-weight substrates (catalase activity) but hinders significantly the protein–protein interactions (the immunogenic and allergenic activities).

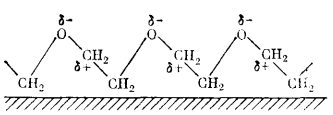
V. THE SURFACE-ACTIVE PROPERTIES OF POLY(ETHYLENE GLYCOL)

The usefulness of PEG for the immobilisation of various physiologically active compounds is also associated with its ability to exert a specific influence on the lipid layer of membranes[44]. The idea of the influence of PEG on the structure of lipids arose as a result of observations that PEG induces the merging of plant protoplasts[45], chicken erythrocytes, chicken erythrocytes with yeast protoplasts[46], and mammalian cells[47].

In order to investigate the molecular mechanism of the action of PEG on cells, a study has been made[44] of the influence of PEG on phospholipid monolayers simulating cell membranes. The authors considered how relations of the surface pressure–area and surface potential–area type vary for phosphatidylcholine and phosphatidylethanolamine monolayers when various low-molecular-weight compounds (glycerol, sorbitol, and dimethyl sulphoxide) as well as PEG specimens with different molecular weights are introduced into the aqueous phase. It was found that all the compounds tested caused considerable changes in the surface potential of the phospholipid layer in the state of dense molecular packing. However, the orders of magnitude of the concentrations of the additive causing the maximum decrease of the surface potential are significantly different for the low-molecular-weight compounds and PEG. Thus the optimum concentrations of the additives in the case of monolayers of dipaltimoylglycerylphosphorylcholine are 3.8 M glycerol, 3 M dimethyl sulphoxide, 1.5 M sucrose, and 2 M sorbitol. On the other hand, the corresponding concentrations of different PEG fractions are 4.2×10^{3} M PEG-6000, 1.5×10^{-3} M PEG-15000, 2.5×10^{7} M PEG-300000, and 1×10^{9} M PEG-5000000. Thus, together with the much greater specificity of the action of PEG, one may note a significant dependence of its influence on its molecular weight.

The authors[44] do not reach a definite conclusion concerning the molecular mechanism of the action of PEG on membranes. In order to account for its role, one should probably turn to the properties of PEG at the interface, primarily at the water–air interface. A detailed study has been made of this problem[48] and it has been shown that PEG is able to form monomolecular films on the surface of water. Depending on the contraction pressure, the polymer can exist in two conformations—a looser conformation occupying an area of 28 Å^2 per monomer unit and a compact conformation occupying an area less than 16 Å^2 per unit.

The study of the behaviour of PEG under polarographic conditions[49] showed that it is adsorbed on negatively charged surfaces in such a way that the negatively charged polar atoms of the ether oxygen are directed towards water, while the non-polar $-CH_2-CH_2$ groups are directed to the negatively charged surface of mercury:



(3)

According to Hillson's calculations[49], the area occupied by the monomeric PEG unit, adsorbed on such a surface, is 13 Å^2.

These data suggest that, when PEG interacts with lipid monolayers, type (3) dipole interactions between the PEG units and the dipoles of the polar head of the lipid also take place. If this is the case, then the range of compounds interacting with PEG and capable of forming complexes of the type resembling the polymeric complexes of PEG with polymeric acids[50] can be greatly extended; numerous surface-active compounds, capable of forming monolayers on the surface of water, should be included among them.

On the other hand, the ability of PEG to act on membranes suggests that this property should be manifested not only in the phenomenon of cell fusion but also in other subcellular processes such as endocytosis, exocytosis, etc., which is extremely important for the functioning of physiologically active compounds incorporating PEG fragments.

Interactions of the dipole–dipole type probably constitute the basis of the molecular mechanism of the action of PEG as a stabiliser of hydrophobic sols[23]. It is striking that in this case one also notes a pronounced dependence of the stabilising activity of PEG on its molecular weight.

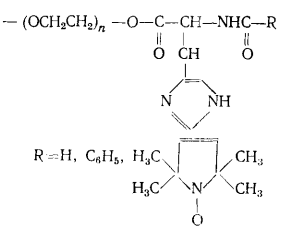
VI. THE SYNTHESIS OF DISUBSTITUTED DERIVATIVES OF POLY(ETHYLENE GLYCOL)

The great practical importance of PEG and the wide-scale employment of this substance and its derivatives in various fields of molecular biology have stimulated the development of research into the chemical modification of the hydroxy-groups at the ends of PEG. We may note that the thoroughly investigated hydroxyethylation reactions, used to synthesise non-ionic detergents[51], make it possible to obtain only monosubstituted PEG derivatives. The most rational method of synthesising bifunctional PEG derivatives consists in the chemical modification of the end groups of PEG.[51,52] Despite their apparent simplicity and triviality, these reactions have in fact many characteristic features and merit a more detailed examination.

PEG specimens with molecular weights up to 20000 are obtained mainly by the hydroxyalkylation method; they contain hydroxy-groups at their ends. Polymers of expoxyethane having a high molecular weight are obtained by other methods; as a rule, they do not contain functional groups at their ends[52].

The hydroxy-groups of PEG undergo reactions of the type usual for alcohols. Numerous PEG derivatives of the type $R-O-(CH_2-CH_2-O)_n-R$, where R = Me, Ph, CH_3CO, or C_6H_5CO, and of the type $R-(CH_2-CH_2-O)_nR$, where R = C, Br, PhS, etc., are known. The reactions between PEG and the corresponding low-molecular-weight reagents are in many cases quantitative and can therefore be used to determine the number-average molecular weight and the molecular-weight distribution[53]. Table 3 presents a list of PEG derivatives substituted in their end groups.

The specific features of the esterification of the hydroxy-groups of PEG were observed for the first time in the synthesis of polymeric derivatives of histidines having the general formula[71]



It was found that in all these cases it is impossible to achieve the complete substitution of the PEG end groups. Special experiments, whose aim was to increase the yield of the polyoxyethylene ethers by modifying the condensing agent and by altering the nature of the solvent and the order of mixing of the reactants, did not lead to a significant alteration of the degree of substitution. All these findings apparently reflect internal features, characteristic of the PEG chain, and definite structural changes occurring in the macromolecule on modification of the latter by the given substituents. The introduction of a polar group at the end of the polymer chain, for example, an imidazole or amide group, leads to structure formation, which hinders the complete modification of all the end groups.

Table 3. Poly(ethylene glycol) derivatives.

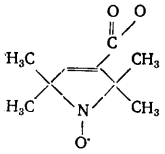
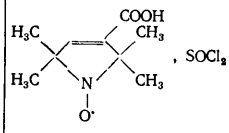
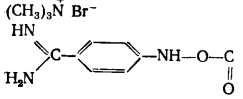
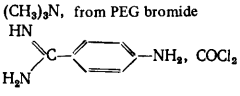
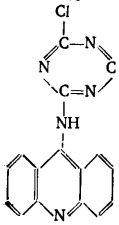
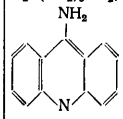
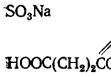
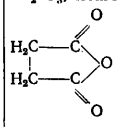
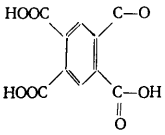
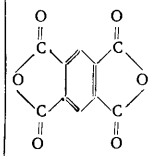
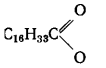
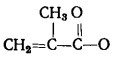
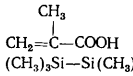
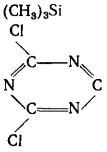
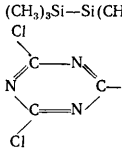
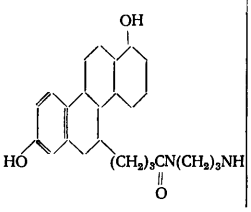
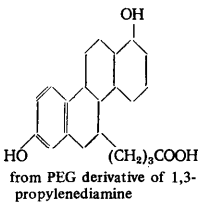
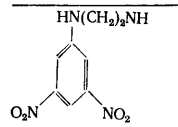
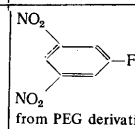
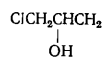
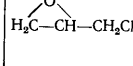
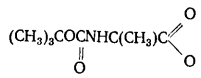
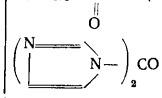
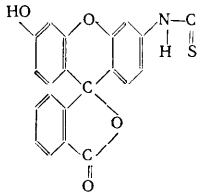
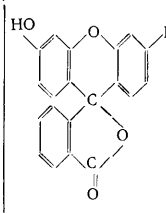
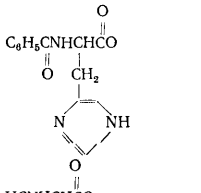
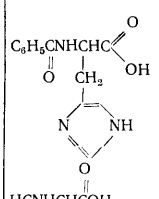
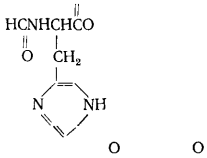
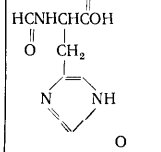
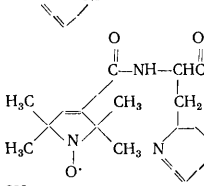
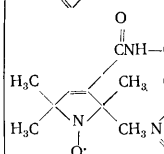
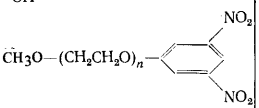
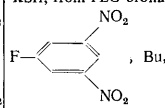
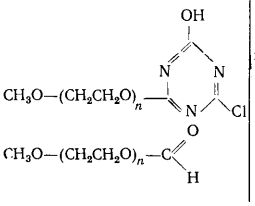
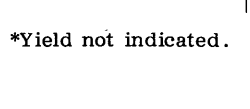
End of group of PEG derivative	Formula of reagent	Yield, %	Refs.
Br	PBr_3	quantitative	29
Cl	$SOCl_2$	quantitative	54
CH_3O	CH_3ONa, from PEG chloride	quantitative	55
C_6H_5O	C_6H_5ONa, from PEG chloride	quantitative	56
C_6H_5S	C_6H_5SNa, from PEG chloride	quantitative	56,57,58
	$(CH_3CO)_2O$	quantitative	59
	CCl_3COCl	quantitative	53
	C_6H_5COCl	quantitative	60
	 , $SOCl_2$	quantitative	61,62
$(CH_3)_3\overset{+}{N}\ Br^-$	$(CH_3)_3N$, from PEG bromide	quantitative	29
	 , $COCl_2$	quantitative	66
$H_2N(CH_2)_2NH$	$NH_2(CH_2)_2NH_2$, from PEG bromide	quantitative	33
$H_2N(CH_2)_3NH$	$H_2N(CH_2)_3NH_2$, from PEG bromide		
	 from PEG derivative of cyanuric chloride	quantitative	67
SO_3Na	Na_2SO_3, from PEG bromide	quantitative	29
		quantitative	68
		quantitative	68
	$C_{16}H_{33}COCl$	quantitative	34,63
		*	37
$(CH_3)_3Si$	$(CH_3)_3Si{-}Si(CH_3)_3$, $(CH_3)_3SiCl$	quantitative	54
		quantitative	64
 from PEG derivative of 1,3-propylenediamine		60	33

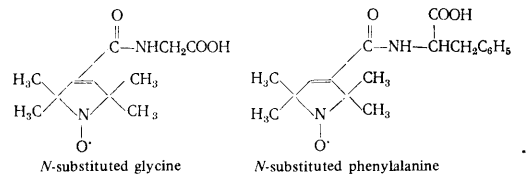
Table 3 (contd.).

End of group of PEG derivative	Formula of reagent	Yield, %	Refs.
	 from PEG derivative of 1,2-ethylenediamine	quantitative	32,38,41
		*	65
	$(CH_3)_3COCNHCH(CH_3)COOH$, 	80	70
		2	6
		37.5	71
		27	72
		8	73
SH	KSH, from PEG bromide	8	74
	 , Bu, Li; from monomethoxy- derivative of PEG	*	39
	reagent not indicated, from monomethoxy-derivative of PEG	*	41
	MnO_2, from monomethoxy-derivative of PEG	*	42

*Yield not indicated.

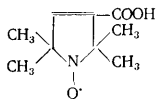
In order to analyse this problem, it is necessary to alter consecutively the nature of the substituent and to follow the changes in the yield and structural characteristics of the

resulting polymers. An attempt has been made[73] at a systematic investigation of the esterification reaction by employing a number of acids RCOOH, where R are aminoacid residues containing the iminoxy-substituent as the *N*-protecting group:



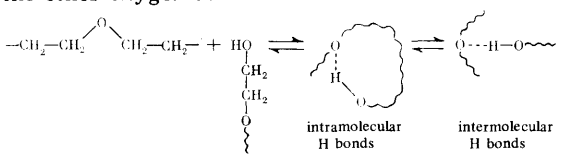
N-substituted glycine *N*-substituted phenylalanine

The simplest spin-labelled acid species, namely the 3-carboxy-2,2,5,5-tetramethylpyrrolin-1-yloxy-radical



was used as the first member of the series. It was shown[73] that the reaction involving the first member of the series goes almost to completion, in agreement with the literature[61]. On passing to the next member, a decrease of the degree of substitution to 35–40% was observed, while in the case of the PEG derivative of phenylalanine the degree of substitution fell to 8%. Thus one may conclude that the introduction of the amide group into the PEG molecule decreases significantly the reaction yield and to a greater extent the more bulky the substituent introduced into the composition of the acid residue.

In order to account for the characteristic features of the reactions described by Topchieva et al.[73], we should consider initially the modification of PEG by carboxylic acids without polar groups. As a rule, such reactions are carried out in non-polar media in which the hydroxy-end groups of the initial PEG form intra- or inter-molecular hydrogen bonds with the ether oxygen atoms of the main chain:

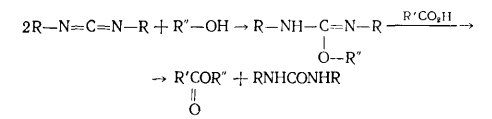


It was shown[60] that, depending on the PEG concentration, one or other type of hydrogen bonds predominate. It was natural to suppose that free hydroxy-groups are involved in the esterification reaction and, as the latter proceeds, the equilibrium shifts towards the non-associated hydroxy-groups.

On the other hand, if the polar group of the substituent is at the end of the macromolecule, then the latter, like the hydroxy-group will tend to form a hydrogen bond (intra- or inter-molecular) with the oxygen atoms of the main chain. The decrease of the degree of substitution on passing to a substituent incorporating polar groups indicates a decrease of the accessibility of the hydroxy-groups of the unreacted PEG. Presumably the hydroxy-groups of PEG, forming hydrogen bonds with the associated polymer molecules, become sterically relatively inaccessible for the reactants.

This hypothesis makes it possible to explain the experimentally observed[73] decrease of the degree of substitution following the introduction of substituents containing polar groups into the ends of the chain, on the one hand, and the dependence of the degree of substitution on the size of the terminal substituent, on the other. Indeed, the greater the bulk of the substituent the looser become the associated species and the greater the number of free hydroxy-groups which can be "entangled" within the associated macromolecules.

In this connection it is of interest to analyse data[34] on the synthesis of a number of polyoxyethylene ethers by the carbodiimide method. The authors[34] obtained an activated PEG derivative by treating it with dicyclohexylcarbodiimide and this derivative was then introduced into a reaction with a carboxylic acid:



The yields of the polymeric esters proved to be very low (Table 1). On the other hand, when palmitoyl chloride is used in the synthesis of the PEG ester of palmitic acid, then esters with a degree of substitution of approximately 80% are obtained. Preliminary activation of PEG by dicyclohexylcarbodiimide apparently leads to the formation of PEG derivatives containing the R–NH–C= group, whose structure is very similar to that of the amide group, which can also form hydrogen bonds leading to the formation of associated macromolecular species. The correlation between the increase in the size of the substituents and the decrease of the degree of substitution of the esters is striking. Quantitative substitution could apparently be attained if dicyclohexylcarbodiimide was used to activate the carboxy-component with subsequent introduction of the glycol.

O–R′

A considerable advance in the development of research into the chemical modification of PEG was made in studies[77,78] devoted to the synthesis of polyoxyethylene esters of aminoacids and peptides[75–78]. In the first place, using a large excess of low-molecular-weight reactants, the authors were able to obtain high yields (80–99%) of *N*-protected aminoacid esters by carrying out the reaction in an aqueous or organic solvent ("the liquid-phase method of synthesis")[77,78]. Next, as a result of the so-called crystallisation method which they developed[70], the authors were able to reduce greatly the duration of the synthesis by increasing the efficiency of the elimination of unreacted substances and impurities from the reaction products. Finally, another advantage of the use of PEG as the *C*-terminal protecting group consists in the fact that the latter facilitates the dissolution of sparingly soluble peptides in solvents of different chemical types, which makes it possible to investigate their conformational state in solutions by the method of circular dichroism[70]. We may note that the possibility of using this method in relation to PEG-peptides is due to the optical transparency of PEG in the visible and ultraviolet regions up to 190 nm.

The hydrophobic octapeptide of the myoglobin sequence 66–73 was investigated by this method[70] and the influence of the substituted or free *N*-terminal amino-group on the conformation of the peptide was observed. It was shown that the blocked octapeptide is more helical than the corresponding peptide with an unsubstituted amino-group. The conformational state of the PEG-esters of nona- and deca-alanines was studied in the same investigation in two solvents—trifluoroethanol and water—and it was shown that the peptide with the free amino-group has the β-conformation in both solvents. The corresponding peptide containing the *N*-protecting group has the helical conformation. Hence the authors[77] concluded that the terminal amino-group has a decisive influence on the formation of the β-structure of the peptide. It is striking that, following the introduction of terminal peptide fragments, capable of forming stable conformational isomers, the effects of the interaction of these groups with the main PEG chain in

organic solvents become minimal. However, in aqueous solutions of PEG-reagents containing hydrophobic end groups there is a possibility of hydrophobic interactions between the non-polar groups of the peptide fragment, leading to the formation of micellar structures in which the hydrophobic nucleus of the micelle consists of the non-polar end groups and the role of the hydrophilic surround is played by the polymeric PEG chains. Association of a similar type has been observed[71] for polyoxyethylene esters of *N*-substituted benzoylhistidines.

In conclusion we should like to emphasize that the study of the interaction of a polymer with biomacromolecules in vitro or with an organism in vivo should develop in parallel with the study of its physicochemical properties. One may hope that this approach will in fact lead to a mutual enrichment of these types of research.

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